Phosphorylation of Ribose and Adenosine in Yeast Extracts.* (18149)

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Yeast extracts are known to ferment ribose and ribose-5-phosphate, the latter at a rate comparable to that of glucose(1). Intact yeast cells can convert added adenosine to adenosine triphosphate (ATP) when inorganic phosphate and a fermentable substrate are also present(2). In the course of an investigation of the metabolism of pentose derivatives in yeast extracts, enzymes have been found which can bring about the phosphorylation of ribose and of adenosine, when ATP is added.

Experimental. Brewers' yeast and bakers' yeast were both supplied by Anheuser-Busch, Inc. The brewers' yeast was collected from a vat at the end of fermentation, and pressed. Upon arrival in the laboratory it was washed and dried according to Lebedev(3) and was stored at 2°C. Maceration juice was prepared by autolysis at 37°C, also according to Lebedev's original instructions. The bakers' yeast was washed several times with tap water and distilled water, and dried at a temperature not exceeding 15°C. Maceration juice was prepared from bakers' yeast by stirring mechanically at 2°C a mixture of 500 g dry yeast, 1150 ml water and 75 ml toluene, over periods of 5 to 7 days. In all cases, solids were removed by high speed centrifugation on a Servall angle-head centrifuge. The reaction mixtures contained, in a final volume of 5.5 ml, 10 to 15 micromoles of the sugar to be tested (or adenosine), 10 to 15 micromoles of ATP, and 0.6 to 0.8 ml of the enzyme solution. All incubations contained 0.006 M NaF and 0.01 M $MgCl_2$, although the need for Mg^{++} has not been definitely established. The reaction mixtures were buffered at pH 7.6 to 8.0 in various experiments, with tris-(hydroxymethyl)aminomethane(4), final concentration 0.025 M. The amine was purified as described elsewhere(5). When changes in phosphorus were to be measured, aliquots were deproteinized by addition of an equal volume of 6% perchloric acid and the filtrates chilled in ice until analyzed. Phosphorus was determined by King's modification of the Fiske and Subbarow method(6), before and after 10 minutes hydrolysis in 1 N H_2SO_4 at 100°. When sugar was to be measured, aliquots were deproteinized with $Ba(OH)_2$ and $ZnSO_4$(7). In this method phosphorylated compounds are adsorbed by the solid phase. Reducing power of the filtrates was measured by the method of Nelson(8).

Evidence for a ribose phosphorylating enzyme is presented in Fig. 1. Curves A, B, and C represent the amount of free ribose actually present in the filtrates. Curve D represents the utilization of ATP, as measured by the disappearance of acid-labile phosphate. The values in curve D are the differences between two parallel reaction mixtures, one with and one without ribose added. Since one deals with relatively small differences between large numbers, this type of determination is less accurate than direct determination of sugar. The first sample in each case was taken at 0.5 minute. The enzyme solu-

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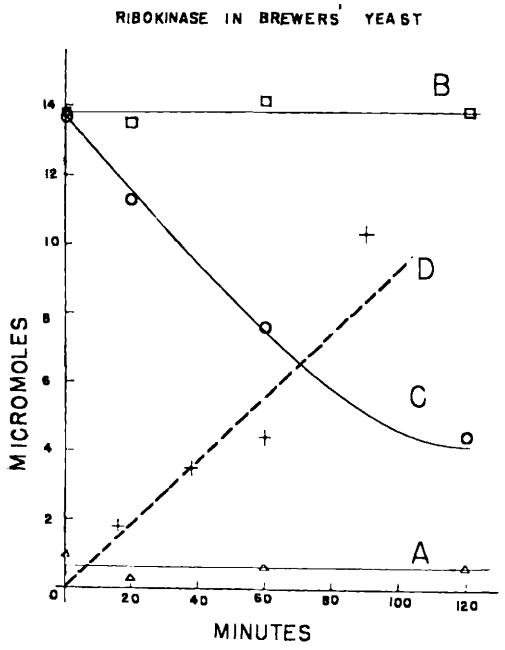

FIG. 1.

Ribokinase in a fraction obtained from brewers' yeast. Curves A, B, and C show utilization of ribose: A—ATP present, ribose omitted; B—ribose present, ATP omitted; C—both ribose and ATP present. Curve D represents utilization of ATP. Details in the text.

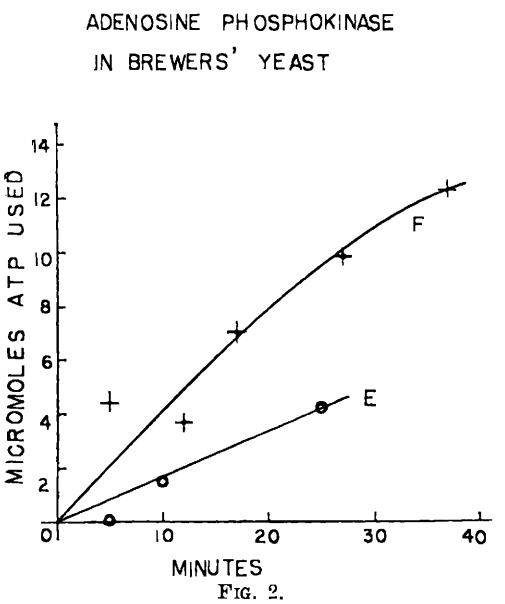

FIG. 2.

Adenosine phosphokinase in brewers' yeast. The data are obtained as in the case of Curve D, Fig. 1, and represent utilization of ATP when adenosine is added. Curve E: Lebedev juice, 18 mg protein added. Curve F: .45–.65 saturated ammonium sulfate fraction, 24.9 mg protein added.

tion in this case was a protein fraction precipitated from brewers' yeast maceration juice with ammonium sulfate, between 0.45 and 0.65 saturation, at pH 7.6. The same protein solution failed to affect D(+)xylose, D(−)arabinose, L(+)arabinose, and D-2-desoxyribose. Because of this specificity the enzyme is designated *ribokinase*. The same extract did phosphorylate glucose at a rapid rate, and also adenosine (Fig. 2). Since a fivefold concentrate of ribokinase from bakers' yeast did not phosphorylate adenosine, it was concluded that a separate enzyme is involved, which is designated as *adenosine phosphokinase*.

Partial purification of ribokinase has been carried out, starting with maceration fluid obtained from bakers' yeast. It was established first that crystalline yeast hexokinase (9) is unable to phosphorylate either ribose or adenosine. Table I shows that at a very early stage one obtains a fraction which is rich in hexokinase and devoid of ribokinase, and another fraction which is relatively enriched in ribokinase and poor in hexokinase. This is conclusive proof that different enzymes are involved. The steps in the purification are as follows:

All operations except the assay are carried out in a cold room at 2°C. The autolysate, pH 6.1, is adjusted to pH 7.5 to 7.6 with dilute ammonium hydroxide. A solution of ammonium sulfate, pH 7.6 and saturated at room temperature is added, 94.3 ml being added per 100 ml autolysate. The resulting solution is 0.485 saturated in ammonium sulfate; it is chilled in ice for ½ hour, and the precipitate which has formed is separated by high speed centrifugation and discarded. The ammonium sulfate concentration is then raised to 0.625 saturation by adding 38.7 ml of the ammonium sulfate solution to each 100 ml of the supernatant solution. The mixture is again chilled in ice for ½ hour and centrifuged. The supernatant fluid is discarded. The precipitated proteins are dis-

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TABLE I. Separation of Ribokinase and Hexokinase in Autolyzed Bakers' Yeast.

Fraction	Total protein, g	Ribokinase* Units/mg	Ribokinase* Total units	Hexokinase† Apparent purity, %
(1) 141 hr autolysate	45.8	0.89	41000	1.87
(2) Ammonium sulfate fraction, .485–.625 saturation	16.0	1.45	23100	2.34
(3) Ppte. at pH 4.6 ionic strength .02	2.6	4.75	12250	0.32
(4) Supernatant fluid of (3)	13.4	Not detectable		4.63‡

* A unit of ribokinase is the amt. of enzyme that would catalyze the phosphorylation of .10 micromol of ribose, at the initial rate observed.

† Hexokinase was measured under optimal conditions (9). The initial rate of the reaction was determined and the number of μg of hexokinase calculated, using the turnover number of 32 μg of glucose phosphorylated per μg of hexokinase per min. The purity of the hexokinase was then calculated from the amt of protein (10) used in the reaction.

‡ The apparent gain in total hexokinase in fraction (4) may be due to the removal of interfering enzymes by the acid precipitation.

solved in the minimum quantity of cold distilled water, and dialysed for 12 to 14 hours against two changes of acetate buffer, pH 6.0, ionic strength 0.05. The volume of buffer should be 200 to 500 times as great as the volume of protein solution. The protein solution is then dialysed for an additional 6 to 8 hours against acetate buffer pH 4.5 to 4.6, ionic strength 0.02. A precipitate begins to form inside the dialysis sac shortly after dialysis against the more acid buffer is begun, and increases in amount. At the end of this dialysis the precipitated protein is collected by centrifugation. The precipitate dissolves with difficulty. It has been found most convenient to suspend the precipitate in a small amount of cold water, adjust the pH to 6.0 with a concentrated solution of sodium acetate, and then add a concentrated solution of sodium chloride, with stirring, until the precipitate dissolves. The final solution is about 0.3 M in salts. Occasionally this precipitate contains some irreversibly denatured protein, which does not dissolve. The protein solution is then adjusted accurately to pH 6.0, ionic strength 0.30, by dialysis against acetate buffer of this concentration, the buffer being changed once in the course of an 8-hour dialysis. The protein solution may now be used for fractionation with alcohol. A solution 80% (by volume) of ethanol in water, ionic strength 0.02 (NaCl) has been used in the present work. The protein solution is chilled in an ice-salt bath and 0.33 volume of the alcohol solution added, the temperature being maintained between $-2°$ and $-5°$C. The solution is then kept at $-2°$ for 5 to 10 minutes, and centrifuged in glass cups, during which time the temperature rises to $+1.5°$. The supernatant fluid is discarded. The precipitate is dissolved by adding 0.5 volumes of concentrated acetate buffer, pH 6, chilled to $-5°$ or lower. The excess alcohol and salts are then removed by dialysis for 4 or 5 hours against a dilute buffer of pH 6.0. A precipitate may form on dialysis. This has never been found to contain any of the desired activity. The final solution is usually a bright yellow.

The redissolved precipitate, obtained after dialysis against acid, retains its potency over a period of months in a freezer box at $-20°$C. The fraction obtained by alcohol treatment does not have such good storage properties. In several experiments, the purification achieved by alcohol treatment in this way has varied between two- and threefold over the previous step. This represents, therefore, a total purification of ten- to fifteenfold over the maceration juice.

In the process of purification, certain interfering enzymes accompany ribokinase. The most highly purified solutions still contain very active phosphatases and the systems that degrade ribose-5-phosphate anaerobically. For this reason, it has not been possible to isolate and to identify the product of the reaction. Paper chromatography of phosphate esters has been carried out successfully by

Hanes and Isherwood(11) and by Benson *et al.*(12). An attempt was made to identify the phosphorylated compound which accumulates during the ribokinase reaction. The reaction was carried out on a scale five times larger than usual; as controls, mixtures were incubated in which ATP was added and ribose omitted, or ribose added and ATP omitted. An additional incubation mixture contained the enzyme solution and authentic ribose-5-phosphate, with no ATP or ribose added. At the end of the incubation period the mixtures were deproteinized with perchloric acid, and the filtrates neutralized. Barium acetate was added in excess, and finally ethanol was added to bring the final concentration to 80%. The precipitated barium salts were collected and dried, resuspended in water and barium removed with sulfuric acid. The supernatant fluids were neutralized with sodium hydroxide and evaporated to small volume at room temperature, and the resultant solutions used for chromatography. The results are summarized in Table II. From the R_f values found it was

TABLE II. Chromatography of Sugars on Filter Paper Strips.

Whatman No. 4 paper was washed with 2N acetic acid and then with 8-hydroxyquinoline in 50% ethanol(11).

Solvent: 25 ml H_2O + 22.5 ml propionic acid + 52.5 ml tert.-butanol, prepared just before use.

Temp.: 29-30°C.

Material	R_f
A. Authentic substances	
Ribose-5-phosphate	.22
L-ketoxylose-1-phosphate	.21
Fructose-6-phosphate	.18
Fructose 1,6-diphosphate	.095
Ribose	.50
B. Incubated materials	
Ribose + ATP	.097
" " alone	None found
ATP alone	" "
Ribose-5-phosphate	.14

The sugars were detected by treating the dried paper with the Folin-Wu sugar reagents, heating for 5 min. at 110°C after applying the alkaline copper solution, and then treating with phosphomolybdic acid(13).

suspected that fructose-6-phosphate or fructose-1, 6-diphosphate were implicated. However, these substances could not be detected in enzymatic tests, using purified enzymes (14-16). Thus the nature of the product is still undecided.

Discussion. The ribokinase reaction (reaction 1) may be one of the important steps in nucleic acid synthesis in yeast, particularly if the product will be found to be ribose-5-phosphate. Some of the early steps in the synthesis may then be represented thus:

ribose + ATP $\xrightarrow{1}$ ribose-5-phosphate $\overset{2}{\leftrightharpoons}$ ribose-1-phosphate + hypoxanthine $\overset{3}{\rightleftharpoons}$ inosine + inorganic phosphate; adenosine + ATP $\xrightarrow{4}$ adenylic acid.

An enzyme catalyzing reaction 2 and designated as *phosphoribomutase* has actually been demonstrated in mammalian tissues(17). This was shown by adding ribose-5-phosphate and hypoxanthine to extracts of rabbit muscle and rat liver, and demonstrating the formation of inosine(18). The existence of phosphoribomutase might have been inferred from the products formed in the anaerobic metabolism of nucleosides and nucleotides (19,20). The yeast extracts used in these studies also contained a potent nucleoside phosphorylase, catalyzing reaction 3. Reaction 1 has not yet been demonstrated in mammalian tissues. Youngburg(21) reported that homogenized kidney cortex could not phosphorylate ribose, and in the present investigation it has been found that a water extract of rat muscle, which contains an

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active glucokinase, is unable to phosphorylate ribose. While reaction 4 probably represents a step in the synthesis of nucleic acid or of additional ATP from adenosine by yeast, mammalian tissues have not yet been found to contain a nucleoside phosphokinase. The extract of rat muscle was tested with adenosine with negative results. Homogenized kidney cortex, prepared as described by Colowick *et al.*(22) was tested with adenosine and guanosine. Nucleoside and inorganic phosphate disappeared and acid labile phosphate accumulated, under aerobic conditions. However, no nucleotides accumulated, and the acid labile phosphate was found to be inorganic pyrophosphate(23). Rapport *et al.* (24) have reported similar findings.

Summary. Yeast autolysates have been found to contain enzymes which catalyze the phosphorylation of ribose and of adenosine, when ATP is added as a phosphate donor. The enzymes, designated ribokinase and adenosine phosphokinase, have been partially purified, but the best preparations still contain large amounts of interfering enzymes, making it impossible to isolate and to identify the products of the reactions. The possible physiological significance of the reactions is discussed.

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Protein Digestibility and Trypsin Inhibitor Activity of Legume Seeds. (18150)

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The poor nutritional quality of some crude legumes has been attributed in early studies to low digestibility(1) and it has been shown that *in vitro* digestibility of crude beans is lower than that of cooked beans(2). Later, factors have been found in a number of crude legumes which inhibit the *in vitro* activity of trypsin(3). However, a purified trypsin inhibitor from soy beans did not inhibit growth of rats and chicks(4). Moreover, no correlation between improvement of growth promoting action of legumes after autoclaving and their trypsin inhibitor content was found in a recent study(5). Some legumes contain heat labile factors which inhibit growth and are probably not identical with trypsin inhibitors(6). No correlation could be detected between growth depression by crude legume seeds and their trypsin inhibitor activity(7). It therefore seemed to be of interest to study the *in vivo* digestibility of some legumes in the crude and autoclaved form and to compare them with their activity to inhibit the action of trypsin *in vitro*.

Experimental. The protein digestibility experiments were made with young Sprague-Dawley rats of about 100 g of weight. For each experiment 2 male and 2 female rats were housed in single screen bottomed cages. Food and water were given *ad libitum*. Food consumption was determined and feces were collected every 3 days, dried at 70°C, weighed, and analysed for N. With the mentioned exceptions, the diets were composed mainly of starch and the amount of the dried and

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