DIFFERENTIATION BETWEEN RIBOSE-3-PHOSPHATE AND RIBOSE-5-PHOSPHATE BY MEANS OF THE ORCINOL-PENTOSE REACTION

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During a study of the energy-rich phosphorus compounds of higher plants, it became necessary to differentiate between ribose-3-phosphate and ribose-5-phosphate. The only method available for distinguishing between these esters at low levels (quantities of 1 mg. or less) is that of Levene and Stiller (1). This method depends on the difference in the rate at which these compounds release their phosphorus in weak acid at 100° over the course of several hours, the phosphorus from the 3-ester splitting off much more readily than that of the 5-ester. This method may be applied not only to the free ribose phosphates, but also to related compounds which possess them. Schlenk (2) used such a procedure to determine the position of the phosphorus on the ribose of diphosphopyridine nucleotide. LePage and Umbreit (3) measured the rate of phosphorus release from the ribose phosphate derived from the adenosine triphosphate of *Thiobacillus thiooxidans*, and concluded, partly on the basis of such data, that this organism possesses an adenosine-3-triphosphate.

The adenylic acids possessing the 3- or 5-ribosephosphate may also be differentiated by several other methods. Klimek and Parnas (4) devised a method based on the formation of a blue soluble complex by the 5-adenylic acid (myoadenylic acid) in alkaline solution in the presence of copper sulfate. Under the same conditions, only an insoluble precipitate is formed by the 3-adenylate (yeast adenylic acid), which after centrifugation leaves a clear colorless supernatant solution. This procedure has recently been more completely standardized by Berlin and Westerberg (5). The most recent method for differentiation between the two types of adenylic acid is that of Kalckar (6), utilizing a purified deaminase first described by Schmidt (7) which removes the amino group from myoadenylic acid, but not from yeast adenylic acid. The reaction is measured spectrophotometrically. The enzyme is without effect on either adenosine di- or triphosphate.

The present paper describes a new method for differentiating between ribose-3-phosphate and ribose-5-phosphate. It is based on a difference in

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the rate of color development of the two esters in the presence of the orcinol reagent. This method may be used not only for the free esters, but for compounds containing them as well. Its chief advantages lie in that it is rapid, can be used for samples containing as little as 10 γ of the esters, and serves as an additional criterion for differentiating between ribose-3-phosphate- and ribose-5-phosphate-containing compounds. It is based on a study of the pentose method proposed by Mejbaum (8) and enables one to resolve a number of discrepancies which appear in the literature.

<div align="center">EXPERIMENTAL</div>

Preparation of Ribose-5-phosphate—Ribose-5-phosphate was prepared from adenosine triphosphate according to the method of LePage and Umbreit (3). The barium salt of adenosine triphosphate was dissolved with 2 N HCl, adjusted to 1 N HCl, heated in a water bath at 100° for 10 minutes, cooled, adjusted to pH 8.2 with 30 per cent NaOH, and the barium phosphate centrifuged down and discarded. The supernatant solution was treated with 4 volumes of alcohol and the barium ribose-5-phosphate collected by centrifugation after several hours in the cold. The preparation, after washing with alcohol and ether, contained only traces of inorganic phosphorus, and no adenine as measured spectrophotometrically. The molar ratio of ribose to organic phosphate was 1.00:1.04.

Preparation of Ribose-3-phosphate—LePage and Umbreit (3) prepared ribose-3-phosphate from yeast adenylic acid in a similar fashion. Several preparations by this procedure from yeast adenylic acid (Schwarz) always yielded material which contained considerable quantities of unhydrolyzed adenylic acid. In one case, after the above procedure, 17 per cent of the adenylic acid had not been split. The entire procedure was repeated on the same sample and yielded a preparation which was 96 per cent pure, the remaining 4 per cent being unhydrolyzed adenylate. The ratio of ribose to organic phosphate (after correction for the adenylic acid remaining) was 1.00:0.98.

Whereas the yields in the case of ribose-5-phosphate were close to theoretical, those of the ribose-3-phosphate were very low. This is understandable in view of the rapid rate of phosphate hydrolysis in the case of the latter (77 per cent is hydrolyzed in 30 minutes in 1 N HCl at 100°) and the apparent relative resistance of the adenine-ribose bond. Indeed Levene and Harris (9) experienced considerable difficulty in preparing the ribose-3-phosphate from guanylic acid by the procedures ordinarily used in the preparation of the 5-phosphate. They attributed this to the greater lability of the 3-phosphate and the difficulty in completely removing nitrogen compounds (presumably adenylic acid).

Preparation of Muscle Adenylic Acid—Muscle adenylic acid, generally prepared by alkaline hydrolysis of barium adenosine triphosphate by the

methods of Lohmann (10), Barrenscheen and Lang (11), or Kerr (12), was in this instance prepared by enzymatic hydrolysis of adenosine triphosphate with the potato pyrophosphatase of Kalckar (13). 122 mg. of barium adenosine triphosphate were suspended in water, dissolved with 2 N HCl, the barium removed with 20 per cent sodium sulfate, and the pH adjusted to 6.0, final volume 3.0 ml. To this were added 5 ml. of 0.2 M acetate buffer, pH 5.5, which contained 10 mg. of $CaCl_2$ per ml., water to 10 ml., and 100 mg. of the lyophilized potato enzyme.[1] The reaction was allowed to proceed at room temperature for 1 hour, at the end of which time it was stopped by the addition of 1 ml. of 100 per cent trichloroacetic acid. The protein was removed by centrifugation, the extract adjusted to pH 8.2, and barium acetate added. The barium-insoluble precipitate was discarded, and the remaining supernatant solution treated with 4 volumes of alcohol. The precipitated barium adenylate was collected after several hours in the cold, washed with 95 per cent alcohol and ether, and dried; 28 mg. of barium adenylate were obtained (40 per cent of theoretical). The ratio of organic phosphorus to pentose to adenine was 1.00:1.07:1.07.

Adenosine triphosphate was prepared as the barium salt by the method of Needham (14). The ratio of total phosphorus to labile phosphorus to pentose was 3.00:1.97:1.03.

Yeast adenylic acid, guanylic acid, and d-ribose were obtained from the Schwarz Laboratories; d-xylose and l-arabinose from the Pfanstiehl Chemical Company.

Phosphorus was determined according to the method of Fiske and Subarrow (15) with Elon as the reducing agent. Pentose was determined according to the procedure of Mejbaum (8) with slight modification. Orcinol (Eastman Kodak) was made up in 95 per cent alcohol, 100 mg. per ml., and added to the $FeCl_3$-HCl solution (0.1 per cent $FeCl_3$ in concentrated HCl) just before use.

Hydrolysis Curves of Ribose-3-phosphate and Ribose-5-phosphate—The phosphorus hydrolysis curves in 0.25 N H_2SO_4 of the ribose-3- and ribose-5-phosphates isolated above are shown in Fig. 1, along with values for yeast adenylic acid. The curves agree well with those reported by Levene and Stiller (1) and Schlenk (2). The ribose-5-phosphate curve is virtually identical with that reported by LePage and Umbreit (3), but that for the 3-phosphate is considerably steeper.

Color Development in Orcinol Reagent—When one heats equal quantities of ribose-3- and ribose-5-phosphates with the orcinol reagent, the final color developed is identical in both cases, but the rates of color development are very different. This is shown graphically in Fig. 2, along with curves for the free ribose, xylose, and arabinose. The average results of a

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[1] We are indebted to Dr. I. C. Gunsalus for this material.

large number of such determinations are shown in Table I, together with their standard deviations.
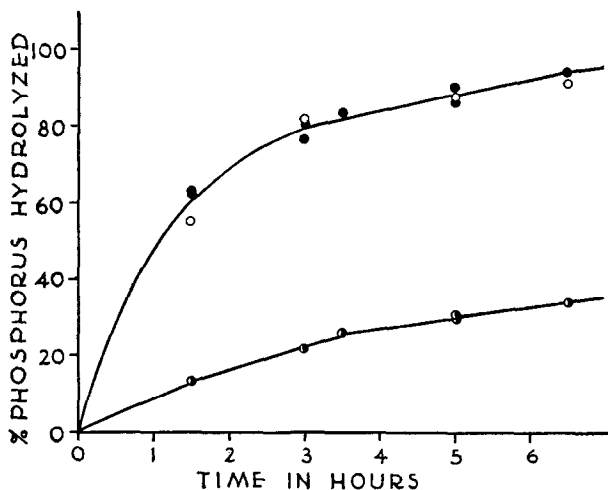

FIG. 1. Phosphorus hydrolysis curves of ribose-3-phosphate (●), yeast adenylic acid (○), and ribose-5-phosphate (◑) in 0.25 N sulfuric acid at 100°.
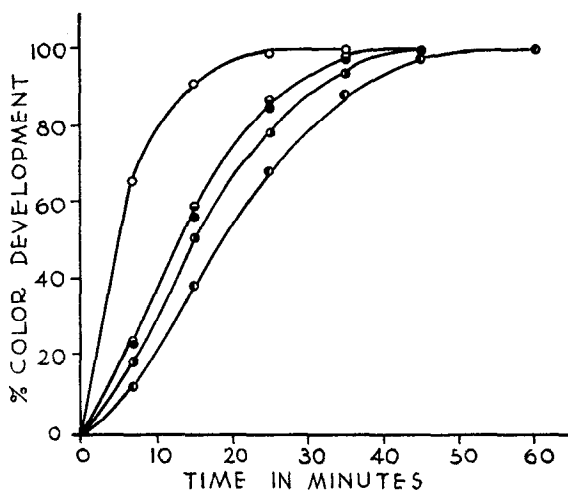

FIG. 2. Rate of color development in the pentose-orcinol reaction. ○ ribose-5-phosphate, ◓ free ribose, ● ribose-3-phosphate, ◑ d-xylose, ◐ l-arabinose.

The experiments were carried out in the following way. Samples containing between 10 and 30 γ of pentose were made to 3 ml. in colorimeter tubes with water. To each were added 3 ml. of the FeCl$_3$-HCl reagent and 0.3 ml. of the alcoholic orcinol. The contents of the tubes

were mixed and read in the Evelyn colorimeter with Filter 660. The tubes were then placed in a boiling water bath and heated for 8 minutes. Since it took approximately 1 minute for the bath to return to boiling, this first reading was called a 7 minute reading. The tubes were removed, cooled, and the color read again. This was repeated at the end of 15, 25, 35, and 45 minutes. The values shown are expressed as per cent of color developed; the 45 minute value was arbitrarily taken as 100 per cent (greater color was not obtained on longer heating, except occasionally with free arabinose (cf. Fig. 2)).

TABLE I

Rate of Color Development in Orcinol-Pentose Reaction

| | No. of deter- mina- tions | Per cent color development | | | | |
| | | Time of heating at 100° | | | | |
		7 min.	15 min.	25 min.	35 min.	45 min.
Ribose-3-phos- phate	36	$26.5 \pm 3.7^*$	62.0 ± 4.5	86.5 ± 3.2	97.0 ± 1.6	100.0
Ribose-5-phos- phate	26	65.5 ± 2.6	90.9 ± 2.0	99.2 ± 0.9	100.0	
Yeast adenylic acid	8	24.6	61.8	87.9	97.4	100.0
Guanylic acid	6	26.9	63.1	88.7	98.1	100.0
Muscle adenylic acid	6	66.8	91.8	99.2	100.0	
Adenosine triphos- phate	6	62.4	88.5	99.1	100.0	

$$* \sigma = \sqrt{\frac{\Sigma d^2}{n}} .$$

In the case of the ribose-5-phosphate, maximum color is often developed at the end of 25 minutes; the 35 minute value is generally the same, whereas the 45 minute value is usually slightly lower. In these cases the per cent color developed is calculated from the maximum value.

It will be noted that complete color is not developed in 20 minutes, the standard heating time used by Mejbaum (8), even in the case of the ribose-5-phosphate. The value is close enough to 100 per cent, however, (95 per cent) to introduce only a small error in any calculations involving ribose in ribose-5-phosphate, especially if ribose-5-phosphate is used as a standard.

Ribose-3-phosphate, on the other hand, behaves quite differently. Its curve is almost identical with that of free ribose, and in 20 minutes shows only about 75 per cent of maximum color development. Xylose and arabinose develop their color even more slowly. Any calculations on the pentose content of ribose-5-phosphate, with the free pentoses as standards,

at the end of a 20 minute heating time will therefore yield values for pentose which are too high, the degree of error introduced depending on the pentose used as a standard.   Calculations involving the 3-phosphates, on the other hand, will give correct values for ribose, but values which are too high with either xylose or arabinose.   All this may be avoided by using a 45 minute heating time, at which time all the sugars develop maximum color.   It is apparent that if one wishes to distinguish between the ribose-3-phosphate and the ribose-5-phosphate, one may conveniently do so by reading the color at 7 minutes and at 45 minutes.   In the case of the 3-phosphate on the average only 26.5 per cent of the final color is developed in this time, whereas for the 5-phosphate, 65.5 per cent develops.
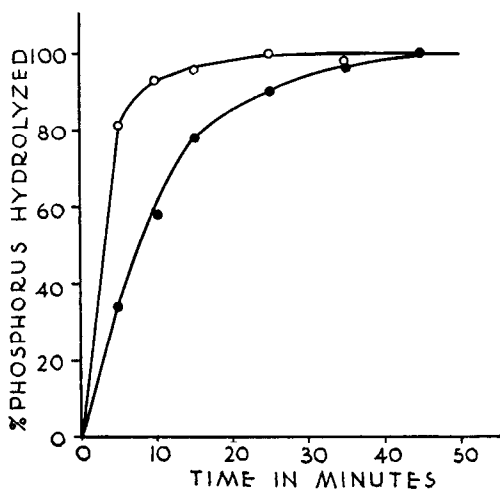

FIG. 3. Phosphorus hydrolysis curves of ribose-3-phosphate (O) and ribose-5-phosphate (●) in 6 N HCl at 100°.

That this method may be applied to compounds containing the ribose-3- and ribose-5-phosphate, as well as to the free esters, is shown in Table I where color development data are presented for yeast adenylic acid, guanylic acid, adenosine triphosphate, and muscle adenylic acid, as well as for the free esters.

Phosphorus Hydrolysis from Ribose-3-phosphate and Ribose-5-phosphate in 6 N HCl—The data presented above show that the color development curves for the ribose-3-phosphate and compounds containing it (yeast adenylic and guanylic acids) are similar to that of free ribose.   It appeared that this might be associated with the greater lability of the phosphorus in ribose-3-phosphate.   Phosphorus hydrolysis curves were therefore run in 6 N HCl (the final acid normality in which the orcinol reaction is carried out).   The results are shown in Fig. 3.   It will be noted that apparently 90 per cent of the phosphorus is split from ribose-3-phosphate in 7 minutes;

under the same conditions less than 50 per cent of the phosphorus is hydro-
lyzed from the 5-phosphate. In the case of the 3-phosphate, therefore,
one rapidly obtains free ribose; one would therefore expect a curve similar
to that of the free sugar. In the case of the 5-phosphate, the greater
stability of the phosphate in some way is related to the more rapid develop-
ment of color.

Effect of Polysaccharide on Rate of Color Development—When one uses
the above method for pure compounds, the results are excellent. If,
however, crude plant or bacterial extracts which contain polysaccharides
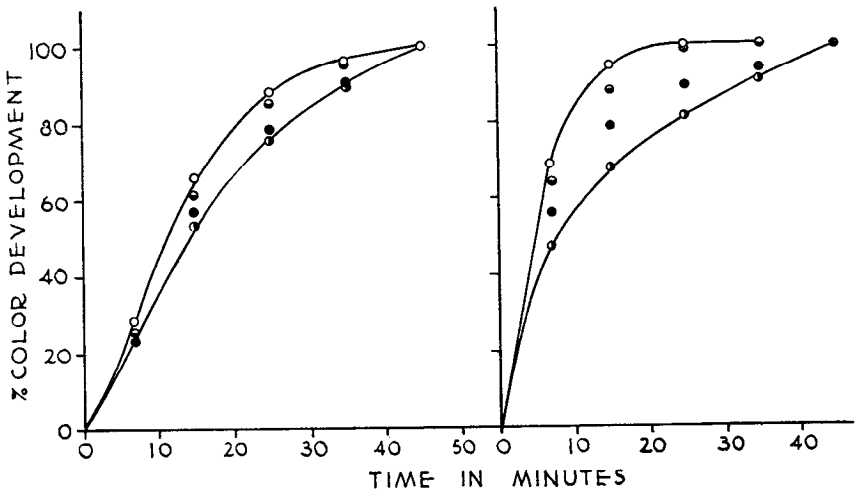and their partially split-products are used, it is difficult to obtain curves

FIG. 4. Influence of soluble corn polysaccharide (I) on the rate of color development
in the pentose-orcinol reaction. Left-hand curves, ribose-3-phosphate; right-hand
curves, ribose-5-phosphate. All samples contain 20 γ of the free esters. ○ no other
additions, ◒ + 50 γ of polysaccharide, ● + 100 γ of polysaccharide, ◗ + 200 γ of
polysaccharide.

which are exactly like those of ribose-3- and ribose-5-phosphates. Under
such conditions, one obtains curves which are low   The effects of polysac-
charide on the color development curve are shown in Fig. 4. In each case,
20 γ of the phosphate esters were used, 50, 100, and 200 γ of soluble corn
polysaccharide, prepared by Sumner and Somers (15), being added.[2]
Similar results are obtained with starch.

DISCUSSION

It is apparent that, by the method described, ribose-5-phosphate can be
detected in pure compounds. The data of this paper also offer a reasonable
explanation for a variety of discrepancies which occur in the literature.

[2] We are indebted to Dr. G. F. Somers for a supply of this material.

Kerr and Seraidarian (16), using free pentoses as standards, report 120 to 125 per cent color development with the 5-phosphates (muscle adenylic and ribose-5-phosphates) which, since these authors used a 20 minute heating time, is entirely in accord with the data of Fig. 2. Mejbaum (8) used 20 minutes heating, but specifically designed the method for determining pentose in the 5-phosphates and used crystalline inosinic acid (muscle) as a standard. Her remarks on the color produced by free pentoses are ambiguous. Schlenk (2) employed a 30 minute heating time and claims agreement among all materials used, which would be roughly, although not exactly, correct. It is apparent that the differences in rate of color development were known in the early literature (4) but apparently had not been studied in sufficient detail to reveal the facts recorded here.

SUMMARY

A method is presented for distinguishing between ribose-3-phosphate and ribose-5-phosphate, based on the rate of color development in the orcinol-pentose reaction.

With this method it is possible to differentiate not only the free esters, but compounds which possess them.

The curve obtained for ribose-3-phosphate is virtually identical with that of free ribose. This is apparently due to the very rapid removal of the phosphorus from ribose-3-phosphates in the strong acid in which the tests are carried out.

The method cannot be used precisely on crude plant and bacterial extracts containing polysaccharides, since these alter the rate of color development.

The bearing of these findings on a number of difficulties in pentose analysis which appear in the literature are discussed.

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