The glycosidic bond between ribose (or deoxyribose) and an imidazole nitrogen is a linkage characteristic of a number of important biological substances, particularly the nucleosides, nucleotides, and nucleic acids. Enzymes which attack the ribosidic linkage have been described by various investigators. Although a direct splitting of the ribosidic linkage of nucleotides has been reported (1), the best studied enzymes are those which liberate purines from purine nucleosides, the so called nucleosidases (2, 3).

Klein (3) investigated purine nucleosidase from spleen and found that the enzyme was inactivated by dialysis. Upon the addition of phosphate or arsenate the activity was restored; the mechanism of this reactivation was not elucidated.

In a recent note (4) an explanation was offered for the function of phosphate in the nucleosidase system. The data indicated that inorganic phosphate disappeared during the fission of inosine, and it seemed evident from this that nucleosidase is a phosphorolytic rather than a hydrolytic enzyme. The following equation was proposed for the reaction (4), ribose-1-purine + phosphate $\rightleftharpoons$ ribose-1-phosphate + purine. The enzyme catalyzing the reaction has been called nucleoside phosphorylase in analogy with Cori's polysaccharide phosphorylase and Doudoroff's disaccharide phosphorylase.

The purpose of the present paper is to describe in greater detail the properties of the enzyme involved in this reaction, the effects of inorganic phosphate and arsenate, the isolation and properties of ribose-1-phosphate, and the reaction equilibria.

**Purine Nucleoside Phosphorylase**

This enzyme was obtained from rat liver and was purified by methods described in a previous paper (5). The enzyme is highly soluble in water, giving completely clear solutions. The activity vanishes after a 5 to 6 hour dialysis against water but can be reconstituted by the addition of...
inorganic phosphate or arsenate. The same is true for much longer periods of dialysis against redistilled water. However, prolonged dialysis (8 or 10 hours) against ordinary distilled water (carried in pipes) results in complete inactivation which cannot be reversed with phosphate or with phosphate plus boiled fresh enzyme (i.e. a coenzyme does not appear to be involved). Klein (3) also observed complete inactivation on prolonged dialysis, and it seems likely that traces of heavy metals are responsible. The most highly purified nucleoside phosphorylase preparations liberated 2 mg. of hypoxanthine per hour per mg. of protein at 25°. This is probably a minimal value, owing to the use of a low substrate concentration.

**Fig. 1. Liberation of hypoxanthine from inosine in the presence of nucleoside phosphorylase with and without added phosphate (P).**

**Effect of Inorganic Phosphate and Arsenate**—Fig. 1 illustrates the effect of the addition of a small amount of phosphate to a mixture of inosine and nucleoside phosphorylase. During the incubation aliquots of the reaction mixture were removed and analyzed for free hypoxanthine by means of xanthine oxidase (6). The salient feature is a 10-fold increase in the amount of liberated purine resulting from the addition of only 25 μg of P per ml. An additional observation was an abrupt cessation of the reaction after a short period of incubation before all of the inosine had been converted to hypoxanthine. The reaction proceeded further when more inorganic phosphate was added. Arsenate had somewhat the same effect as phosphate, although the reaction was slower with arsenate. In an experiment in which 100 μg of inosine were incubated 30 minutes with nucleoside phosphorylase and varying amounts of arsenate, the results

1 The arsenate used was Baker's analyzed.
were as follows: without arsenate no hypoxanthine was liberated; with 10 γ of arsenate less than 0.5 γ of hypoxanthine was found; with 100 γ of arsenate 6 γ of hypoxanthine were liberated.

The simplest interpretation of the experiment illustrated in Fig. 1 is that phosphate is combined in organic form during the fission of inosine. If this is true, it should be possible to demonstrate a decrease in inorganic phosphate proportional to the liberation of purine. Such an uptake of phosphate could not be demonstrated with the strongly acid reagents ordinarily used for the measurement of inorganic phosphate. However, when inorganic phosphate was determined according to the method of Lowry and Lopez (7) at pH 4 it was possible to show (1) that phosphate was taken up during the enzymatic splitting of purine ribosides, and (2) that a highly acid-labile phosphoric ester was formed. A typical protocol

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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</table>

Phosphorolysis of Guanosine and Formation of Equivalent Amount of Labile Phosphorus Compound

5 micromoles of guanosine plus 3.6 micromoles of phosphate incubated with liver nucleoside phosphorylase containing guanase. Temperature 30°, pH 7.5.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Inorganic P</th>
<th>Inorganic + labile P</th>
<th>Labile P formed</th>
<th>Guanine liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>micromoles</td>
<td>micromoles</td>
<td>micromole</td>
<td>micromole</td>
</tr>
<tr>
<td>0</td>
<td>3.48</td>
<td>3.52</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3.31</td>
<td>3.64</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>3.02</td>
<td>3.69</td>
<td>0.67</td>
<td>0.71</td>
</tr>
<tr>
<td>40</td>
<td>2.53</td>
<td>3.28</td>
<td>0.75</td>
<td>0.79</td>
</tr>
</tbody>
</table>

is given in Table I. In this case the substrate was guanosine. Similar results are obtained with inosine.

The procedure used for the phosphate analysis is briefly as follows: Aliquots of the enzyme-substrate mixture were cooled to about −5°, deproteinized with perchloric acid, and centrifuged 1 to 2 minutes in a refrigerated centrifuge at 12,000 R.P.M. Immediately, 0.1 and 0.2 ml. of the supernatant were pipetted into 1.5 ml. of 0.5 M acetate-acetic acid buffer, pH 4. This sample was analyzed for inorganic phosphate. The labile phosphate was determined on another aliquot which was allowed to incubate at 30° for 30 minutes before neutralization with acetate-acetic acid buffer. The phosphate color was developed in both samples by adding 0.2 ml. of molybdate (2.5 per cent in 0.1 N H₂SO₄) and 0.2 ml. of ascorbic acid (0.75 per cent in 1 M sodium acetate). The color was measured in the spectrophotometer at 700 μμ.

The guanine was estimated from the xanthine formed during the incubation, with an optical procedure previously described (6).
Ribose-1-phosphate

Preparation—Ribose-1-phosphate was prepared by enzymatic splitting of a nucleoside and subsequent purification. The substrate, 40 to 60 mg. of inosine, was dissolved in 1 ml. of 1 M dipotassium phosphate. Purified xanthine oxidase (dialyzed free of sulfate ions) was added in excess (5) in order to oxidize and thus remove the hypoxanthine liberated. Crystalline catalase, prepared by dioxane fractionation according to Sumner and Dounce (8) (about 100 \( \gamma \) of protein), was added to prevent accumulation of hydrogen peroxide. In order to prevent later difficulties in the barium fractionation the nucleoside phosphorylase was freed from sulfate ions by adding barium acetate and the excess barium was removed with phosphate buffer. 5 to 6 ml. of this nucleoside phosphorylase were added to the other components, and after saturation with oxygen, the mixture was pipetted into three Warburg-Barcroft manometer vessels and incubated at 30°.

The consumption of oxygen was observed during the incubation as a measure of the progress of the reaction. For each micromole of hypoxanthine liberated and converted to uric acid, 1 micromole of oxygen (22.4 c.mm.) should be consumed. After 3 or 4 hours of incubation one-third to one-half of the inosine had been split and the purine had been oxidized by the xanthine oxidase to uric acid, which precipitated out. The samples from all of the vessels were pooled, chilled to \(-5^\circ\), and the bulky precipitate which consisted mainly of uric acid, together with some hypoxanthine or xanthine, was centrifuged off. To the chilled supernatant was added cold perchloric acid (final concentration 2 per cent), the acidified mixture was immediately centrifuged at \(-5^\circ\) for not more than 2 minutes at 12,000 R.P.M. in a refrigerated centrifuge, and the protein-free filtrate was quickly neutralized by the addition of concentrated sodium hydroxide. When the acid mixture was kept below 0° and neutralized not longer than 3 or 4 minutes after the addition of acid, the loss of ribose-1-phosphate through hydrolysis did not exceed 15 per cent. To the neutralized filtrate were added 8 or 10 volumes of cold acetone; the precipitate formed was dried and then extracted with 3 or 4 ml. of ice-cold water. The free purines remained largely in the residue and the solution contained the salts, inosine, ribose-1-phosphate, and free ribose. To this solution was then added barium acetate in excess, the pH was adjusted to 8, and an equal volume of ethanol was added. The barium precipitate thus formed was washed once with 50 per cent ethanol. The supernatant plus the washing contained the inosine and the main part of the free ribose. The barium precipitate contained inorganic phosphate and ribose-1-phosphate. The precipitate was dried in a stream of air for 10 or 15 minutes to remove ethanol and was then extracted with 4 or 5 ml. of warm
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water (30–35°) for a period of 15 to 20 minutes in a mechanical shaker, and then centrifuged. The supernatant contained appreciable amounts of barium ribose-1-phosphate besides some barium phosphate. The barium precipitate was reextracted twice with water, as described, and the supernatants were pooled, the combined extracts were concentrated in vacuo at low temperature, and the precipitate of barium phosphate was removed in the centrifuge. By addition of an equal volume of ethanol, ribose-1-phosphate precipitated out. The yield of ribose-1-phosphate present was determined by measuring the amount of labile phosphate in the sample (see below). The yields obtained were generally low, ranging from 7 to 10 mg. of barium ribose-1-phosphate from 40 to 60 mg. of inosine. For example, in one experiment with 45 mg. of inosine the oxygen uptake indicated that about 15 mg. of inosine had been split, corresponding to 20 to 22 mg. of barium ribose-1-phosphate, or only one-third of the theoretical amount. There are probably several reasons for the comparatively low yields. Besides losses due to acid hydrolysis and to amounts retained in the bulky barium phosphate precipitate, a large loss undoubtedly occurs during the incubation. The generation of ribose-1-phosphate proceeds slowly and the degradation of the ester due to traces of phosphatases, specific or non-specific in the enzyme preparation, is probably of appreciable proportions. Thus it has been found that incubation times of 5 or 6 hours give still lower yields than 3 hours, particularly if the oxygen consumption drops to very low values during the last hours of incubation. Whether ribose-1-phosphate participates in side reactions with other nitrogenous compounds besides purines remains to be seen.

Samples of barium ribose-1-phosphate were subjected to the following analyses: reduction capacity before and after hydrolysis by means of the iodometric method of MacLeod and Robison (9); labile phosphorus by the method of Lowry and Lopez (7); and total pentose after hydrolysis by the orcinol method (10).

1 ml. of the solution analyzed was found to contain 5.86 micromoles of total aldopentose, 3.86 micromoles of which appeared upon hydrolysis. The total pentose measured colorimetrically was 5.82 micromoles. 3.32 micromoles of labile phosphorus were found. It can be seen that hydrolysis released approximately 1 equivalent of aldose for each mole of labile P liberated. The aldose content of the unhydrolyzed sample is apparently due to contamination with free ribose.

The results of the analysis furnish strong evidence that the ester formed is an aldopentose-1-phosphate; i.e., an aldopentose with phosphate esterified to the aldose group. It would be desirable to isolate larger amounts of the new phosphoric ester or to obtain it by chemical synthesis in order definitely to establish whether or not its structure is d-ribose-1-phosphate and to
determine whether it is an α or β ester. However, since the ester was formed by the phosphorolysis of a purine riboside, it seems most likely that the new ester is a ribose phosphoric ester, and, until proved otherwise we suggest it be called ribose-1-phosphate.

The hydrolysis curve of ribose-1-phosphate in dilute acid at room temperature has been reported (7). The ester is somewhat more acid-labile than phosphocreatine but less so than acetyl phosphate. In 0.5 N sulfuric acid it was found that the half time for splitting was 4 minutes for phosphocreatine, 30 to 40 seconds for acetyl phosphate, and 2.5 minutes for ribose-1-phosphate. The rate of splitting in dilute acids was found to be increased about 30 per cent by the presence of molybdate. Molybdate has a much greater influence in accelerating the acid hydrolysis of phosphocreatine and acetyl phosphates (11, 12).

**Enzymatic Synthesis of Hypoxanthine Riboside**

When ribose-1-phosphate and hypoxanthine are incubated together with liver nucleoside phosphorylase, a rapid synthesis of hypoxanthine riboside (inosine) occurs. In order to effect this synthesis it is necessary that inorganic phosphate be removed as thoroughly as possible. This was accomplished by the addition of 0.1 volume of ammoniacal magnesium citrate to a solution of ammonium ribose-1-phosphate. After vigorous stirring for 10 minutes the precipitate was removed and the solution was neutralized to pH 7.5 by the addition of succinic acid.

In a typical experiment 0.58 micromole of ribose-1-phosphate was incubated with 0.57 micromole of hypoxanthine in the presence of nucleoside phosphorylase (100 γ of protein). After 30 minutes, samples were chilled to −5°, deproteinized, and analyzed by optical methods previously described (6). The hypoxanthine was measured by changes in the ultraviolet absorption when oxidized with xanthine oxidase. Inosine was determined by further changes in the ultraviolet absorption when inorganic phosphate and nucleosidase were also added. In a control sample, in which ribose-1-phosphate was hydrolyzed prior to addition, the hypoxanthine remained entirely in the free form. When all of the components were present, the free hypoxanthine was reduced from 0.57 to 0.11 micromole and 0.45 micromole of inosine was synthesized. Fig. 2 records the actual ultraviolet extinction changes in the purine analysis of this experiment. Glucose-1-phosphate was inactive in the test.

If ribose-1-phosphate is added in excess to a solution of hypoxanthine and incubated in the presence of liver nucleoside phosphorylase, almost all of the free hypoxanthine disappears and is found as inosine (Table II). It can be seen that less than 5 per cent of the hypoxanthine remains after incubation with an excess of ribose-1-phosphate. Table II also shows that xanthine does not react with ribose-1-phosphate.
In Fig. 3 the equilibrium reaction between equimolar amounts of ribose-1-phosphate and hypoxanthine or between equimolar amounts of ribose-

![Graph showing changes in optical density with time](image)

**Fig. 2.** Analysis for free and ribosidic bound hypoxanthine in filtrates from mixtures of equimolar amounts of ribose-1-phosphate and hypoxanthine incubated with liver nucleoside phosphorylase. In the controls the component omitted was added after deproteinization. O, control, incubated without hypoxanthine; X, control, incubated with the acid hydrolysis products of ribose-1-phosphate substituted for the ribose-1-phosphate; □, complete sample; for the inosine analysis 50 μl of inorganic phosphate were added; ●, the same with 300 μl of inorganic phosphate; Xa., addition of purified xanthine oxidase for the determination of free hypoxanthine; Nu., addition of nucleoside phosphorylase plus inorganic phosphate for the determination of ribosidic bound hypoxanthine (inosine).

**Table II**

*Formation of Nucleoside from Hypoxanthine*

<table>
<thead>
<tr>
<th>Purine</th>
<th>Free</th>
<th>Ribosidic bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>micromole</td>
<td>micromole</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Control*</td>
<td>0.196</td>
</tr>
<tr>
<td></td>
<td>Complete system</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Control*</td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>Complete system</td>
<td>0.181</td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.012</td>
<td>0.195</td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*Ribose-1-phosphate omitted.

1-hypoxanthine and phosphate is graphically illustrated. The position of equilibrium favors the formation of hypoxanthine riboside. It appears
from Fig. 3 that with equimolar proportions of the reactants only about 12 per cent of the hypoxanthine remains free. The synthesis proceeds quite rapidly with the amount of enzyme used. The half time in Fig. 3 is a little over 5 minutes.

**Enzymatic Synthesis of Guanosine**

In the experiments dealing with the synthesis of guanine ribosides the techniques were not as adequate as in the case of the synthesis of hypoxanthine ribosides, and the analyses are consequently incomplete, particularly with respect to equilibrium constants. The low solubility of guanine and its tendency to become adsorbed on protein precipitates make it difficult to obtain reliable values for the amount of free guanine present in solution during incubation. However, the amount of guanosine synthesized is readily determined, since this substance is quite soluble in both neutral and acid solution. Therefore, although the value of the equilibrium constant was not obtained, it was possible to demonstrate an *in vitro* synthesis of guanosine. The nucleoside phosphorylase used for the synthesis was freed of impurities of guanase by the procedures mentioned previously. In Table III the molar concentration of guanine is more than double that of ribose-1-phosphate, and since no visible precipitation occurred, the guanine presumably remained in excess. Almost 90 per cent of the ribose-1-phosphate was split and a definite but lesser amount of guanine was converted to nucleoside.
The data show that almost all of the labile phosphate was utilized but that the phosphate did not reappear as inorganic phosphate. It apparently became bound in a more acid-stable linkage. It is noteworthy that in the sample incubated with guanine alone the inorganic phosphate decreased. On the other hand, the sample which was incubated with ribose-1-phosphate alone, although containing the same amount of labile phosphate, showed much more inorganic phosphate. In other words, the presence of guanine in some way caused an uptake of inorganic phosphate into a rather acid-stable organic linkage. The nature of this phosphorylation remains unknown. It is also difficult to explain at the present time the fact that more than 1 micromole of labile phosphate disappeared for each micromole of guanine synthesized as guanosine.

**Table III**

*Enzymatic Synthesis of Guanosine from Guanine and Ribose-1-phosphate*

Complete system, 1 micromole of guanine, 0.5 micromole of ribose-1-phosphate, incubated 30 minutes at 30° with nucleoside phosphorylase. In the control experiments the missing component was added after deproteinization.

<table>
<thead>
<tr>
<th></th>
<th>Inorganic P</th>
<th>Inorganic + labile P</th>
<th>Labile P</th>
<th>Change in labile P</th>
<th>Guanine bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>micromoles</td>
<td>micromoles</td>
<td>micromole</td>
<td>micromole</td>
<td>micromole</td>
</tr>
<tr>
<td>Ribose-1-phosphate + enzyme</td>
<td>1.47</td>
<td>1.95</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine + enzyme</td>
<td>1.21</td>
<td>1.65</td>
<td>0.44</td>
<td>0.04</td>
<td>0</td>
</tr>
<tr>
<td>Ribose-1-phosphate + guanine + enzyme</td>
<td>1.23</td>
<td>1.29</td>
<td>0.06</td>
<td>0.42</td>
<td>0.27</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The phosphorolysis of inosine and guanosine can be described as a reversible exchange of purine for phosphate and *vice versa* according to the equation, ribose-1-purine + phosphate ⇌ ribose-1-phosphate + purine. This type of reaction is analogous to that described by Cori and his group (13) in the formation of glucosidic linked hexose chains in polysaccharide or in the formation of sucrose as described by Doudoroff (14).

The synthesis and phosphorolysis of nucleosidic linkages (nitrogen ribosidic linkages) is probably a reaction of wide significance. There is reason to believe that desoxyribosides may participate in the same type of equilibrium (3). Of interest in this connection is also the fact that, certain microorganisms require ribosides as growth factors. Thus *Hemophilus influenzae* cannot use free nicotinamide but only nicotinamide nucleotides (15) or nicotinamide nucleoside (16). A certain x-ray mutant of *Neurospora* requires pyrimidine riboside but cannot grow on free pyrimidine plus ribose (17).

The phosphorolysis of purine nucleosides may also play an important rôle in the nucleic acids. Colowick and Price (18, 19) have recently shown...
the existence of an enzyme in skeletal muscle which in the presence of inorganic phosphate liberates guanine from ribonucleic acid. Apparently a reversible enzymatic reaction takes place by which guanine is exchanged for phosphate.

SUMMARY

1. Nucleoside phosphorylase has been prepared from rat liver. The enzyme, which is a highly water-soluble protein, is inactivated by dialysis. If the dialysis is performed against redistilled water, the activity can be completely restored by the addition of inorganic phosphate or arsenate.

2. When a purine nucleoside (inosine or guanosine) is split by nucleoside phosphorylase, 1 mole of phosphate is bound into an acid-labile organic linkage for each mole of purine liberated.

3. The barium salt of a highly acid-labile ester has been isolated as a reaction product of nucleoside phosphorylase action and has been identified as an aldopentose with the phosphate group bound to the aldehyde group. On the basis of origin and properties the ester is thought to be ribose-1-phosphate. It can be obtained by the enzymatic splitting of either inosine or guanosine.

4. When ribose-1-phosphate is incubated with hypoxanthine or guanine in the presence of liver nucleoside phosphorylase, a rapid synthesis of purine nucleoside takes place. It has been demonstrated that during the formation of hypoxanthine riboside 1 mole of hypoxanthine is bound to ribose for each mole of phosphate liberated from ribose-1-phosphate. The position of equilibrium favors the formation of hypoxanthine riboside.

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