DEGRADATION OF CERTAIN NUCLEOTIDES BY HAEMOPHILUS INFLUENZAE AND HAEMOPHILUS AEGYPTIUS

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Received for publication July 6, 1959

Little attention has been paid to the degradation of nucleotides by the genus Haemophilus although Lwoff and Lwoff (1937a, b) and McIlwain and Hughes (1948) demonstrated that Haemophilus parainfluenzae was capable of destroying diphosphopyridine nucleotide (DPN). More recently, Kalckar (1954) reported the occurrence of a specific nucleotide pyrophosphatase and associated nucleotide phosphatase in sonic extracts of Haemophilus influenzae which hydrolyzed uridine diphosphoglucone to glucose 1-phosphate, uridine, and inorganic phosphate. The present work demonstrates that in addition to uridine diphosphoglucone, several other nucleotides, including DPN, are degraded by sonic extracts of H. influenzae and Haemophilus aegyptius.

MATERIALS

Bacterial materials. H. aegyptius strain 180a (Pittman) and H. influenzae strain b 747 (Pittman) were newly recovered from the freeze-dried state. The organisms were grown in beef heart infusion broth containing 1 per cent Fildes peptic digest of blood. As soon as heavy growth developed (about 7 hr), the culture was diluted 1 to 100 in beef heart infusion broth and 5 ml were seeded in Blake bottles on beef heart infusion agar containing 3 per cent blood digest. The bottles were incubated at 35 C for 16 hr. Purity was determined by examination of a stained smear. The bacteria were then washed from the medium with cold saline, centrifuged, resuspended in cold saline, and collected by centrifuga-

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tion at 5 C. The packed cells were then suspended in a small volume of cold distilled water and disrupted by sonic vibration in a 9 kc sonic vibrator for at least 20 min. From a heavy streak of the sonic extract on a blood agar plate only a few colonies developed. The extract was then freeze-dried or held frozen at -20 C until used. No viable cells were recovered after either procedure.

Enzyme preparation. Bacterial extracts were fractionated by adding four volumes of ammonium sulfate solution (saturated at 0 C and adjusted to pH 8 with ammonium hydroxide). The precipitate was collected by centrifuging, then dissolved in and dialyzed at 2 C for 3 hr in a continuous flow, rocking dialyzer against 0.02 M sodium acetate buffer, pH 6.0, containing $10^{-3}$ M ethylenediaminetetraacetate in cellulose tubing (treated according to Tsuboi and Hudson, 1955) containing a few glass beads for mixing.

The dialyzed preparation was treated with 2 per cent protamine sulfate in two steps of 0.1 mg each per initial 1.0 mg enzyme protein. The ratio of the optical densities at 250 and 260 mµ approached 1.0. Purification of uridine diphosphoglucose degrading activity at this stage was roughly 3- to 5-fold. Further attempts to separate this activity from 5'-nucleotide phosphatase activity were unsuccessful. Crude and fractionated preparations remained fully active for more than 2 years while stored at -20 C, and withstood repeated freezing and thawing. Insoluble material was centrifuged off at various times without loss of activity.

Analyses and materials. Reaction mixtures contained 0.1 M glycine buffer, pH 9.0, 3 to $5 \times 10^{-2}$ M MgCl₂, 90 µg per ml and 115 µg per ml enzyme preparation from H. influenzae and H. aegyptius, respectively, and substrate as indicated. Total substrate or product change was routinely determined as the difference between the amount found at 0 time and after 30 min incubation at
requirement from 5'-adenosine destroyed pH dations shown and zyme pyridine nucleotide of man A from semen of (Heppel 1951b). Uridine diphosphoglucone was determined with uridine diphosphoglucone dehydrogenase (Strominger et al., 1957). DPN was determined with alcohol dehydrogenase (Colowick et al., 1951) and reduced DPN (DPNH) with lactic dehydrogenase (Kornberg and Pricer, 1950). The intact ribosylpyridinium bond of DPN was estimated with KCN (Colowick et al., 1951). Triphosphopyridine nucleotide (TPN) was measured using glucose 6-phosphate dehydrogenase (Kornberg and Ho-recker, 1955). Inorganic phosphate was determined by the method of Gomori (1942). Protein was estimated by the method of Lowry et al. (1951).

Yeast inorganic pyrophosphatase preparations were generously supplied by Dr. Leon Heppel of National Institutes of Health and Dr. Jack Preiss of Duke University (Heppel and Hilmo, 1951a). A 5'-nucleotide phosphatase was prepared from bull semen in collaboration with Drs. Hilmo and Heppel (Heppel and Hilmo, 1951b). Diphosphopyridine nucleotide pyrophosphorylase was purified from hog liver acetone powder by a slight modification of the procedure of Kornberg (1950).

Nucleotides were chromatographed on Whatman no. 1 paper using the neutral ethanol-1 m ammonium acetate (7.5:3) solvent mixture of Paladini and Leloir (1952).

Nucleotides were purchased from the Sigma Chemical Company, St. Louis, Missouri.

RESULTS

As shown in Table 1, both uridine and adenosine pyrophosphate-containing nucleotides were degraded by metal dependent activities in the enzyme preparations obtained from H. influenzae and H. aegyptius. The optimal pH for the degradations shown in Table 1 was found to be near pH 9.0, except for TPN which was most rapidly destroyed at pH 8.0.

Also shown for comparison of rates is the metal requirement for formation of inorganic phosphate from 5'-adenosine monophosphate. Phosphate was also released from uridine diphosphoglucose, DPN, and from several other 5'-phosphate compounds tested, including uridine tri-, di-, and mono-phosphates, adenosine triphosphate, inosine diphosphate, cytidine monophosphate, and the 2', and 3'-uridine monophosphates, but not from flavine mononucleotide. A magnesium requiring inorganic pyrophosphatase activity was also observed in extracts of H. aegyptius.

Products. The following experiments were done to establish the pathway by which uridine diphosphoglucose and DPN were degraded by these enzyme preparations.

(1) Uridine diphosphoglucose:—Glucose 1-phosphate formation could be continuously followed by spectrophotometric assay in the absence of added inorganic pyrophosphate; nor was it inhibited by preincubation with yeast inorganic pyrophosphatase. These results indicate a hydrolytic rather than pyrophosphorolytic cleavage of uridine diphosphoglucose. A balance experiment indicated recovery of 0.12 μmole of glucose 1-phosphate and 0.03 μmoles inorganic phosphate from 0.13 μmole substrate. Samples of deproteinized reaction mixtures taken at various times during incubation were chromatographed and examined under ultraviolet light, revealing that uridylic acid was first formed and then degraded, resulting in the formation of uridine. These results are quite in agreement with those previously reported by Kalckar (1954).

(2) Diphosphopyridine nucleotide:—DPN degradation did not result in appreciable loss of the KCN reactive pyridinium riboside bond, indicating little nucleosidase activity. The destruction of DPN also occurred in the absence of added inorganic pyrophosphate and in the presence of yeast inorganic pyrophosphatase.

Evidence suggesting hydrolytic cleavage of the pyrophosphate moiety of DPN was obtained by demonstration of 5'-nucleotide formation in the following experiment with H. influenzae. A reaction mixture was incubated until 1.65 μmoles DPN were completely digested, with concomitant release of 0.62 μmole inorganic phosphate. Two volumes of ethanol were added to deproteininate and the alcohol removed by evaporation after centrifuging. Samples were treated with bovine seminal plasma 5'-nucleotide phosphatase which releases phosphate from 5'-nucleotides. Inorganic phosphate was released and amounted to 2.03 μmoles, which indicated that adenosine 5'-phos-
phosphate and nicotinamide riboside 5' phosphate were products of DPN degradation. The formation of nicotinamide riboside 5' phosphate was further demonstrated by using samples of the DPN degradation products to resynthesize DPN in the presence of the hog liver DPN pyrophosphorylase system. As shown in table 2, no resynthesis occurred in the absence of adenosine triphosphate, and recovery of the nicotinamide mononucleotide was about 65 per cent of theory.

* Inhibition of uridine diphosphoglucose degradation. The data in table 3 indicate that the uridine pyrophosphate compound is protected from destruction by the H. influenzae enzymes in the presence of DPN and by nucleotides containing phosphate esterified to ribose at the 5', but not the 2' or 3' positions, nor by glucose-1-phosphate.

Uridinediphosphoglucose or DPN degradation

TABLE 2

<table>
<thead>
<tr>
<th>Addition of Adenosine Triphosphate*</th>
<th>DPN Synthesized</th>
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<tr>
<td>Time</td>
<td>min</td>
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<td></td>
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</tbody>
</table>

* Reaction mixtures contained aliquot of 1.65 μmoles of DPN-haemophilus degradation products, purified hog liver DPN-pyrophosphorylase, 5 μmoles MgCl₂, 50 μmoles tris(hydroxymethyl)-aminomethane, pH 7.4, yeast inorganic pyrophosphatase, and water in 0.5 ml.

† DPN expected: 0.32 μmole. DPN determined on 0.05-ml samples with alcohol dehydrogenase in 0.5-ml assay volume at times shown.

TABLE 3

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Additions</th>
<th>Uridine Diphosphoglucose Δ*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μ mole</td>
</tr>
<tr>
<td>1</td>
<td>Uridine diphosphoglucose</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>+ Diphosphopyridine nucleotide</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>+ Glucose 1-phosphate</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>+ 5'-Uridine monophosphate</td>
<td>1.25</td>
</tr>
<tr>
<td>2</td>
<td>Uridine diphosphoglucose</td>
<td>0.115</td>
</tr>
<tr>
<td></td>
<td>+ 5'-Uridine monophosphate</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>+ 3'-Adenosine monophosphate</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>+ 3'-Uridine monophosphate</td>
<td>1.20</td>
</tr>
</tbody>
</table>

* Uridine diphosphoglucose assay.
was inhibited 50 per cent or more by the presence of $10^{-2}$ M orthophosphate and tetraborate or $10^{-3}$ M fluoride, inorganic pyrophosphate, and ethylenediaminetetraacetate.

**DISCUSSION**

The data presented suggest that a hydrolytic cleavage of the pyrophosphate moiety with resultant formation of 5'-nucleoside monophosphates, is the initial step in the degradation of nucleotide pyrophosphate compounds by the enzyme preparations made from sonic extracts of *H. influenzae*. Examination of sonic extracts of *H. aegyptius* have indicated the presence of similar nucleotide degrading activities. This is not surprising since recent reports indicate a widespread distribution of metallic ion dependent DPN pyrophosphatase and 5'-nucleotide phosphatase activities among bacteria (Swartz et al., 1956; and Stadtman, 1957).

Of interest is the observation that degradation of uridine diphosphoglucose by the enzymes obtained from *H. influenzae* was inhibited by DPN and other nucleoside 5'-phosphates. The DPN inhibition might indicate that both nucleotide pyrophosphate containing compounds are split by the same nucleotide pyrophosphatase. However, the inhibition by 5'-monophosphates suggest that perhaps the inhibition is merely a way of regulating activity of the uridine diphosphoglucose pyrophosphatase. Further work is necessary to clarify these points.

**ACKNOWLEDGMENTS**

The authors would like to express appreciation to Dr. Herman M. Kalckar, in whose laboratory these experiments were started, and for many valuable discussions during the course of this work. The technical assistance of Mr. James F. Marshall in the bacterial work is gratefully acknowledged.

**SUMMARY**

Stable enzyme preparations made from sonic extracts of *Haemophilus aegyptius* and *Haemophilus influenzae* (type b) were found to exhibit several metallic ion dependent alkaline nucleotide pyrophosphatase and nucleotide phosphatase activities.

**REFERENCES**


NUCLEOTIDE DEGRADATION BY HAEMOPHILUS


