DIARYL PYROPHOSPHATASE AND FAD PYROPHOSPHATASE

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(Received for publication, June 6, 1956)

It was reported (1) that diphenyl pyrophosphate could be hydrolysed by inorganic pyrophosphatase into 2 moles of monophenyl phosphate when a cofactor present in a heated kidney autolysate is added. The present experiment has shown that diphenyl pyrophosphate can be split at the pyrophosphate linkage by a liver enzyme preparation not containing inorganic pyrophosphatase. The action of this enzyme, diaryl pyrophosphatase, when purified, is specific to diphenyl pyrophosphate. It is inactive to flavin adenine dinucleotide, which, however, is hydrolysed by muscle enzyme to produce flavin monophosphate and adenylic acid. The latter enzyme seems to belong to the nucleotide pyrophosphatase of Kornberg (2) and is inactive to diphenyl pyrophosphate. The preparation and properties of these enzyme solutions are described here.

EXPERIMENTAL AND RESULTS

Measurement of Hydrolysis at Pyrophosphate Linkage—To estimate the extent of diphenyl pyrophosphate hydrolysis at the pyrophosphate linkage, the monophenyl phosphate produced should be quantitatively split by monoesterase into phenol and inorganic phosphate which are colorimetrically measurable by the indophenol method (3) and Fiske-Subbarow’s or Youngburg’s method, respectively. As an effective monoesterase a human urine sample simply dialysed was used. Activity of urine monoesterase at its optimum pH 5 was strong enough to completely hydrolyse monophenyl phosphate of 0.001 M concentration in two hours, whereas in the same conditions inorganic and diphenyl pyrophosphates used as substrates remained intact. Potassium diphenyl pyrophosphate was prepared according to the procedure of Neuberg and Wagner (4).
Preparation of FAD Solution—It was prepared from guinea pig liver according to the warm water extraction method of Yagi (5) and the phenol-ether method of Warburg and Christian (6). A slight modification was made in following points. The liver extract for phenol extraction of flavin compounds was obtained by heating liver slices in a mixture (1:5) of acetate buffer (pH 5) and physiological saline solution for 5 minutes at 80°, homogenising the coagulated tissue debris, heating again for 5 minutes, and centrifuging and the supernatant fluid was, prior to phenol treatment, saturated with sodium chloride instead of ammonium sulfate, since the contamination of the latter caused an inhibition of enzymatic FAD hydrolysis. Total flavin content of the solution thus prepared was estimated by the method of Fujita-Yagi (5) after conversion to lumiflavin. Flavin compounds remained in the water layer after three times extraction with benzyl alcohol were assumed to be flavin mononucleotide (FMN) and FAD. For estimation of FAD, the solution was incubated at pH 5 with urine monoesterase for five hours to hydrolyse FMN to flavin riboside (FR), then extracted three times with benzyl alcohol to make free from FR, and the FAD remained was fluorometrically estimated by the method of Fujita-Yagi (5) after conversion to lumiflavin. The complete hydrolysability of FMN under the conditions mentioned above was ascertained from the result that FMN (Takeda) solution incubated with monoesterase and treated with benzyl alcohol did not produce any lumiflavin. FAD suffered no decrease by urine enzyme. FAD content of the solution thus prepared and used for the experiments was 3 to 5 µg. per ml. and a trace of FMN and FR was present.

The Test of FAD Pyrophosphatase Activity—It was carried out as in the case of diphenyl pyrophosphatase in two steps: Two ml. of FAD solution, 2 ml. of FAD pyrophosphatase, and 2 ml. of buffer solution were mixed in a brown glass tube, covered with toluene, and incubated for the time as indicated in the experimental results. For removal of FMN at the second step of the procedure, the mixture was adjusted to pH 5 by adding 0.1 N NaOH or acetic acid, 1 ml. of acetate buffer of pH 5 was added, and the volume was made to 9 ml. with water. It was heated at 80° for 15 minutes to stop the enzyme action and then incubated with 2 ml. urine monoesterase solution at 37° for 2 hours. FR produced was removed by benzyl alcohol extraction and FAD remaining unhydrolysed was estimated fluorometrically after conversion to lumiflavin.
Preparation of Liver Diaryl Pyrophosphatase—Pig liver brei was autolysed with 2 volumes of water at 37° for 3 days and the filtrate was dialysed for 2 days against running water. This solution which contained monoesterase and inorganic and diaryl pyrophosphatases was acidified to pH 5 by addition of dilute acetic acid, the protein precipitated was centrifuged off, and the supernatant solution was neutralized to pH 9 by addition of 0.1 N NaOH, heated at 65° for ten minutes, and dialysed. The solution thus treated did not hydrolyse monophenyl phosphate and inorganic pyrophosphate in pH range from 3.2 to 9.0. It caused by its single action in the same pH range no liberation of phenol or inorganic phosphate from diphenyl pyrophosphate. However, when the diphenyl pyrophosphate solution incubated for some time with the enzyme solution was heated at pH 5 and 100° for five minutes to inactivate any enzyme present in the test mixture and then reincubated with urine monoesterase, the production of equivalent amounts of free phenol and inorganic phosphate was observed.

Optimum pH of Diaryl Pyrophosphatase—It was measured by the two-step procedure. The presence of two isodynamic, acid and alkaline, types was thereby revealed (Table I). Calcium or magnesium ion showed neither activation nor inhibition of the liver diaryl pyrophosphatase.

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<th>pH</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Hydrolysis per cent</td>
<td>9</td>
<td>18</td>
<td>13</td>
<td>12</td>
<td>16</td>
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Mixture of 1 ml. M/200 diphenyl pyrophosphate, 2 ml. of diluted diaryl pyrophosphatase, and 2 ml. buffer (M/10 acetate or Veronal) was incubated for two hours. After adjusting to pH 5, 1 ml. of acetate buffer of pH 5 was added and the total volume was made to 8 ml. by the addition of water. The mixture was heated for 5 minutes at 100°, cooled to 37°, and incubated with 2 ml. urine monoesterase for two hours. Phenol liberation was measured. Production of 2 moles phenol corresponds to 100 per cent hydrolysis.

Liver diaryl pyrophosphatase solution so long as treated as described above, still contained an alkaline FAD pyrophosphatase, since two ml. of it completely hydrolysed 11 µg. FAD at pH 8 to 9 to FMN.
in 2.5 hours, while at pH 5 the hydrolysis proceeded to the extent of 50 per cent, and at pH 3 and 4, almost none. The diaryl pyrophosphatase solution could be made free from this alkaline FAD pyrophosphatase as follows.

**Purification of Diaryl Pyrophosphatase**—The enzyme solution heated at pH 9 as mentioned above was acidified with 0.1 N hydrochloric acid to pH 3 and heated for 10 minutes in a water bath of 75°. After adjusting to pH 5.6 by the addition of 0.1 N NaOH and leaving in an ice-box over night, the precipitate produced was suspended in 0.05 M Veronal buffer of pH 9, kept at 37° for 3 hours, and centrifuged. The clear supernatant solution obtained was proved to retain the diaryl pyrophosphatase action and presented 52 per cent and 46 per cent hydrolysis of diphenyl pyrophosphate in two hours at pH 5 and 8, respectively, but showed no FAD hydrolysis at all.

From the results mentioned above it should be concluded that diaryl pyrophosphatase and FAD pyrophosphatase are different enzymes. Then preparation of FAD pyrophosphatase free from diaryl pyrophosphatase activity was desirable.

**Muscle FAD Pyrophosphatase**—Rabbit muscle was autolysed with 2 volumes of water at 37° for 4 days and the filtrate was dialysed. This solution was unable to produce any phenol from diphenyl pyrophosphate at pH 5 and 9, even after successive action at pH 5 of urine monoesterase, and therefore, it was free from diaryl pyrophosphatase. However, it exhibited, in case of no further purification, some activity of monoesterase and inorganic pyrophosphatase at pH 5 and 9. Since it has been proved that alkaline FAD pyrophosphatase of the liver was resistant to heating for 10 minutes at 65° and pH 9, the muscle autolysate was treated in a similar way. The solution thus heated, neutralised, and dialysed did not contain any monoesterase and inorganic pyrophosphatase. Optimum pH of FAD hydrolysis was 8 as illustrated in Table II. Alka-

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<tbody>
<tr>
<td>Hydrolysis per cent</td>
<td>3</td>
<td>5</td>
<td>16</td>
<td>17</td>
<td>100</td>
<td>100</td>
<td>78</td>
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Two ml. of FAD solution (4.6 µg. per ml.) was used. Reaction time for the first step at various pH was 18 hours. Reaction time for the second step with urine monoesterase at pH 5 was 5 hours.
line FAD pyrophosphatase activity was not influenced by Ca or Mg ions.

*Paper Chromatography of Hydrolytic Products of FAD*—FAD solution was incubated with muscle FAD enzyme at pH 8 for 24 hours. After adjusting to pH 5, it was saturated with ammonium sulfate, extracted with phenol, and riboflavin compounds in the phenol layer were transferred as usual to 0.1 ml. of water for chromatography. Toyo filter paper No. 50 and the upper layer of a mixture of n-butanol, acetic acid, and water (4:1:5) was used. No fluorescent spot of FAD (Rf 0.02) was visible under a mercury lamp and instead, a very intense spot of FMN (Rf 0.09) appeared. However, when the FAD hydrolysate was successively incubated with urine monoesterase and then concentrated by the phenol procedure, a single fluorescent spot was observed at Rf 0.30, indicating the presence of riboflavin produced by hydrolysis of FMN, while no spots of other Rf were visible. The production of FMN from FAD was thus verified. If FAD were to be split at the pyrophosphate linkage, adenylic acid should be the other product, which could be detected by the following procedure. Ten aliquot samples of FAD hydrolysate of pH 8 by muscle FAD pyrophosphatase were acidified to pH 5 and heated at 80° for 15 minutes. The combined filtrate was concentrated to 1 ml. at 80° for application to paper chromatography. Under an ultraviolet irradiation with the Matsuzaki's apparatus (7), a dark spot of adenosine 5'-phosphate was visible, Rf of which was 0.65 and 0.71, when developed with a mixture of 5 per cent potassium dihydrogen phosphate and isoamyl alcohol and of 5 per cent disodium hydrogen phosphate and isoamyl alcohol, respectively.

*Action of Potato Phosphatase on Diphenyl Pyrophosphate and FAD*—Kornberg (2) isolated nucleotide pyrophosphatase from potatos. In the present experiment, the purification of potato enzymes according to his method remained at the first fractionation with ammonium sulfate and the precipitate obtained was dissolved in water and dialysed. This enzyme solution hydrolysed diphenyl pyrophosphate at the optimum pH of 3. Besides this acid enzyme there were, however, an acid monoesterase and two, acid and alkaline, inorganic pyrophosphatase. The solution was, therefore, heated as usual at 65° and pH 9 for 10 minutes and dialysed. Hydrolysis of FAD by this dialysed solution proceeded optimally at pH 3, the higher the pH of the reaction mixture the more FAD remained unchanged.
Diphenyl pyrophosphate was hydrolysed into two moles of monophenyl phosphate by an enzyme, provisionally named diaryl pyrophosphatase, without any liberation of free phenol. Successive action of urine monoesterase produced phenol and inorganic phosphate in equivalent amounts. Phosphodiesterase, monoesterase, and inorganic pyrophosphatase had no action on that substrate. Kurata (1) reported twenty years ago that the inorganic pyrophosphatase could hydrolyse diphenyl pyrophosphate when an activator was present. The diaryl pyrophosphatase in the present experiment was free from inorganic pyrophosphatase and seemed to need no activator. Whether there are two different enzymes for the hydrolysis of pyrophosphate linkage of diphenyl pyrophosphate remains at present undetermined. The nomenclature of diaryl pyrophosphatase was preferred, since it was inactive to FAD. There are acid and alkaline diaryl pyrophosphatases in the liver, the optimum pH being 4 and 8, respectively. The acid enzyme free from the alkaline isodynamic enzyme can be prepared from a potato extract.

FAD was, on the other hand, hydrolysable by a specific FAD pyrophosphatase. An alkaline FAD pyrophosphatase of the muscle could be prepared free from other phosphatases, whereas an acid FAD pyrophosphatase was found in a potato extract. It can be said that there are also two isodynamic types of FAD pyrophosphatase.

Since diaryl and FAD pyrophosphatases are different from each other, specificity of the enzymes capable of splitting pyrophosphate linkage depends upon the sort of hydroxyl compounds esterified by phosphoric acid. The results reported by Kornberg (2) that DPN, TPN, and FAD are hydrolysed by a single nucleotide pyrophosphatase is reasonable. According to this author the optimum pH of DPN hydrolysis by potato enzyme is 8 and that of FAD hydrolysis seems to be near this pH range. In the present experiment, however, an acid FAD pyrophosphatase was found in a potato extract. Then it may be said that there are in plants two isodynamic FAD pyrophosphatase as in animals.

The FAD hydrolysis by this enzyme proceeded specifically at the pyrophosphate linkage just as in the case of diphenyl pyrophosphate by diaryl pyrophosphatase. Since FMN and adenylic acid were identified as the products, the formation of any intermediate such as flavin pyrophosphate or flavin pyrophospho-ribose could be excluded.
SUMMARY

Diphenyl pyrophosphate is hydrolysed by diaryl pyrophosphatase into two moles of monophenyl phosphate. This enzyme is inactive to flavin adenine dinucleotide, which is in turn split by nucleotide pyrophosphatase to produce flavin monophosphate and adenylic acid. For either diaryl pyrophosphatase or nucleotide pyrophosphatase there are two isodynamic, acid and alkaline, enzymes. The acid FAD pyrophosphatase is found in potato extract and the alkaline enzyme in muscle autolysate. Acid diaryl pyrophosphatase contained in potato extract can be obtained free from isodynamic alkaline enzyme, whereas the two isodynamic diaryl pyrophosphatases of the liver, though they could be made free from other phosphatases, are incapable at present, of being separated from each other.

This work was supported in part by a grant from the Ministry of Education, given to Prof. S. Akamatsu, the director of this department.

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