Evidence for Alkylphosphorylation of Tyrosyl Residues of Stem Bromelain by Diisopropylphosphorofluoridate*

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ABSTRACT: Stem bromelain was known to be phosphorylated by diisopropylphosphorofluoridate (DFP) without inhibition of the caseinolytic activity. With diisopropylphosphoryl (DIP) enzyme thus formed, a study has been made of the site of alkylphosphorylation in the enzyme molecule. Unlike DIP-chymotrypsin, DIP-stem bromelain failed to give rise to *O*-phosphorylserine upon acid hydrolysis in 2 N HCl at 100° for 18 hr. A phosphorus-containing amino acid was isolated from the digest of DIP-stem bromelain with *Streptomyces griseus* and *Bacillus subtilis* proteinases, and this compound was identified with an authentic specimen to be *O*-DIP-L-tyrosine. The pattern of difference spectra of DIP-stem bromelain *vs.* stem bromelain was found to be almost identical with that of *O*-DIP-L-tyrosine *vs.* L-tyrosine. From the observed difference in molar absorbancy at 277 mμ between DIP-enzyme and the reference enzyme preparation the number of tyrosyl residues modified per molecule of DIP-enzyme was calculated.

A decrease in number of ionizable phenolic hydroxyl groups of DIP-stem bromelain was also calculated from measurements of absorption at 295 mμ in 0.01 N NaOH. These spectral data were found to be in good agreement with the results of phosphorus analysis. From these findings, it is concluded that the alkylphosphorylation of stem bromelain has occurred exclusively at the phenolic hydroxyl groups of tyrosyl residues of the enzyme protein. The alkylphosphorylation of tyrosyl residues of stem bromelain is not a surprising phenomenon in view of the known reactivity of a phenolic hydroxyl group of tyrosine with DFP.

It was shown in the preceding communication (Murachi and Yasui, 1965) that DFP phosphorylates stem bromelain without affecting the proteinase activity. The fact that the phosphorylation accompanies no inhibition is the most striking feature of this reaction in contrast to the well-known reactions of DFP with chymotrypsin and some other hydrolases, whereby phosphorylation and inactivation simultaneously occur with a cause and effect relationship (see, for example, the review by Hartley, 1960). Besides, the phosphorylation by DFP of stem bromelain differs from that of chymotrypsin in many other respects such as the number of moles of phosphorus incorporated per mole of enzyme protein, the rate, and the pH dependence of the reaction. It was suggested that these differences must reflect differences in the mechanism and the rate of phosphorylation between stem bromelain and chymotrypsin (Murachi and Yasui, 1965). The present communication describes the evidence to indicate that DFP alkylphosphorylates stem bromelain exclusively at its tyrosine hydroxyl groups. The evidence was obtained from (1) the absence of *O*-phosphorylserine in the acid hydrolysate of DIP-stem bromelain,[1] (2) the isolation of *O*-DIP-L-tyrosine from the enzymatic hydrolysate, (3) the ultraviolet absorption spectra of DIP-enzyme as compared to those of the untreated enzyme, and (4) the agreement of the results of phosphorus analysis with those of spectral data concerning the number of tyrosyl residues modified.

Phosphorylation by DFP without direct effect on enzymatic activities has also been demonstrated with other enzymes like papain, Taka-amylase A (Murachi, 1963), egg white lysozyme (Murachi and Inagami, 1963), and ficin (Gould *et al.*, 1963). Gould and Liener (1965) have obtained indirect evidence to indicate that the seryl (and/or threonyl) residues of ficin are the most likely sites of phosphorylation. On the other hand, Ashbolt and Rydon (1957) reported that DFP reacts with tyrosine in a slightly alkaline medium to form an *O*-DIP derivative, while no phosphorylation occurs with serine. The latter report seems to be compatible with the present observation that tyrosine hydroxyl groups are the sites of alkylphosphorylation of stem bromelain.

Materials and Methods

Stem Bromelain. The enzyme preparation used was isolated from crude "Bromelain" (lots 181 and 182) from the Hawaiian Pineapple Company, Honolulu,

* From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan. *Received August 2, 1965; revised September 24, 1965.* This work was presented in parts before the 19th International Congress of Pure and Applied Chemistry, July 10–17, 1963, London, England (Murachi and Inagami, 1963), and Symposium on Protein Structure, Chemical Society of Japan, October 27–28, 1963, Tokyo, Japan. This work was supported in part by a grant from the Ministry of Education, Japan, and by a U. S. Public Health Service research grant (GM 08714) from the National Institute of General Medical Sciences.

[1] Abbreviation used: DIP, diisopropylphosphoryl.

Hawaii,[2] by the method of Murachi *et al.* (1964). Fraction 6 was employed for the present experiment. For routine works, the concentration of the native stem bromelain was determined by measuring the absorbancy at 280 mμ with a Hitachi–Perkin-Elmer Model UV-VIS-139 spectrophotometer, using $A_{1\,cm}^{1\%}$ 20.1 and the corresponding molar absorbancy of 6.68×10^4. Lower values, 19.0 for $A_{1\,cm}^{1\%}$ and 6.33×10^4 for molar absorbancy, were reported in a previous communication (Murachi *et al.*, 1964) and used for earlier experiments (Inagami and Murachi, 1963; Murachi and Yasui, 1965). These lower values had been obtained with a Hitachi Model EPB-U spectrophotometer and are still valid so far as the optical measurement is made with this instrument. A newer instrument, Model UV-VIS-139, offers higher wavelength selectivity and has given 3–5% higher absorbancies for solutions of stem bromelain as well as bovine serum albumin over the range 260–290 mμ wavelength. In experiments for difference spectra between the untreated stem bromelain and DIP-enzyme, the protein concentrations were compared on the basis of color values of the biuret reaction (Gornall *et al.*, 1949), using bovine serum albumin as standard. The biuret reaction was the method of choice since this was supposed to remain unaffected after a varying number of tyrosyl residues of the enzyme protein were alkylphosphorylated: both the absorbancy at 280 mμ and the Folin–Ciocalteu reaction would have been affected by the extent of modification of tyrosyl residues.

Mercuri-Stem Bromelain. Stem bromelain was treated with $HgCl_2$ to obtain mercuri-stem bromelain. The treatment was made in order to minimize the autodigestion of the enzyme during the subsequent incubation with DFP. Mercuri-stem bromelain had been known to react as well with DFP as the untreated enzyme (Murachi and Yasui, 1965). Thus, 7.8×10^{-4} M stem bromelain was mixed with 1.6×10^{-3} M $HgCl_2$ at 4° for 2 hr, and the mixture was dialyzed *vs.* 5 l. of water overnight in the cold. The dialyzed solution was used as the mercuri-stem bromelain.

Chymotrypsin. A product (lot 6010 AI) of Worthington Biochemical Corp., Freehold, N. J., was used.

Streptomyces griseus Proteinase. "Pronase P" was obtained from Kaken Chemical Company, Tokyo, Japan. The enzyme was partially purified by the following procedure. The procedure was developed in this laboratory according to a personal suggestion by Dr. Nomoto based on the published method (Nomoto and Narahashi, 1959). All the operations were conducted in the cold. To a solution of 5 g of "Pronase P" in 200 ml of 0.02 M calcium acetate was added 35 g of ammonium sulfate, and the precipitate was removed by centrifugation. To the supernatant fluid was added 12.5 g of ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 33 ml of water. The solution was dialyzed overnight *vs.* 5 l. of 0.1 M sodium acetate containing 0.02 M calcium acetate. The dialyzed solution was applied to a 1.0 × 6 cm column with Duolite A-2 resin, which had been equilibrated with 0.1 M sodium acetate. The enzyme protein that was not adsorbed on the resin was further decolorized with 1 g of charcoal, "Shirasagi" brand (Wako Chemical Co., Osaka, Japan). After dialysis overnight *vs.* 5 l. of 0.1 M sodium acetate containing 0.02 M calcium acetate, the enzyme solution (47 ml) was mixed with an equal volume of cold acetone and the mixture was centrifuged. To the supernatant fluid was added 60 ml of acetone, and after 1 hr the precipitate was collected by centrifugation, dissolved in 20 ml of 0.02 M calcium acetate, and dialyzed overnight as described above. Lyophilization yielded 1 g of the enzyme. The specific activity toward casein of the partially purified proteinase was found to be 1.6 times as high as that of "Pronase P." The partially purified preparation contained no inorganic and organic phosphorus.

Bacillus subtilis Proteinase. The crystalline enzyme was obtained from Nagase Company, Osaka, Japan, and used without further purification. The enzyme preparation contained no phosphorus.

DFP. A product of Sumitomo Chemical Industries, Co., Ltd., Osaka, Japan,[3] was used for the present experiment. As was reported in a preceding communication (Murachi and Yasui, 1965), the Sumitomo DFP gave no inhibition of the caseinolytic activity of stem bromelain. Ten microliters of DFP preparation was assumed to correspond to 55 μmoles.

O-DIP-L-Tyrosine. An authentic specimen of *O*-DIP-L-tyrosine was supplied by Dr. Rydon (see Ashbolt and Rydon, 1957). The compound was also synthesized in this laboratory from L-tyrosine and DFP by a procedure essentially the same as that described by Ashbolt and Rydon (1957). The purification of the crude product included ion-exchange chromatography on Amberlite IR-120 with 2 N NH_4OH as eluant. Recrystallization from water gave white needles, mp 168–169° (uncor.); lit. mp 167–168° (Ashbolt and Rydon, 1957). Phosphorus analysis and ninhydrin color value of the synthesized compound revealed the presence of 0.96 mole of phosphorus/mole of free amino group as calculated in terms of L-tyrosine equivalent. The *N*-2,4-dinitrophenyl derivative of the synthesized compound had mp 160.5–161.5° (uncor.); lit. mp 159–160° (Ashbolt and Rydon, 1957). The synthesized compound showed identical R_F values and electrophoretic mobility on paper with those of the specimen supplied by Dr. Rydon. (For the solvent systems used, see Table IV below.)

O-Phosphoryl-DL-Serine. The compound was synthesized from DL-serine and phosphorus oxychloride according to the method of Neuhaus and Korkes (1958).

Ultraviolet Absorption. Measurements were made on a Hitachi–Perkin-Elmer Model UV-VIS-139 spectro-

[2] We are indebted to Dr. Ralph M. Heinicke for generous supply of this material.

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TABLE I: Reaction of DFP with Stem Bromelain and Chymotrypsin under Various Conditions.[a]

Enzyme and Sample No.	Method of Reaction	Incubation Mixture (ml)	Enzyme Protein (mg)	DFP Added (mmoles)	Moles DFP/Mole Protein	Incubation Period (hours)	Incubation Temp (degrees)	Yield of DIP-enzyme (mg)	Moles P Found/Mole Enzyme	Relative Spec. Activity toward Casein
Stem bromelain										
10	1	10	350	1.65[b]	155	3	30	325	1.50	1.02
14	1	100	2400	5.5[c]	75.6	5	30	1900	1.00	0.98
48	2	50	450	None	0	3	25	398	0.00	1.00[d]
49	2	50	430	1.1	77.4	3	25	406	1.49	1.00
50	2	50	440	2.75	193	3	25	402	2.97	0.97
51	2	50	430	5.5	387	3	25	328	3.87	1.03
Chymotrypsin										
7	1	10	250	0.11[b]	11	1	30	190	0.93	0.00

[a] In Method 1, the enzyme was incubated with DFP added in portions and the pH of the incubation mixture was maintained at pH 8.2 by occasional additions of alkali. In Method 2, the mercuri-enzyme was reacted with DFP and the pH was maintained at 8.2 by the use of a pH-Stat assembly with alkali as titrant. [b] DFP was added in three portions. [c] DFP was added in five portions. [d] The specific activity of this preparation toward casein was taken as unity.

photometer; 10-mm quartz cells were used. The temperature was in a range between 17 and 20°. Symbols used in this communication are: ϵ, molar absorbancy; $\Delta\epsilon$, difference in molar absorbancy.

Other Methods. The pH was measured with a Hitachi Model M-4 glass electrode pH meter except as otherwise indicated. For gel filtration Sephadex G-25, medium, obtained from Pharmacia, Uppsala, Sweden, was used. Phosphorus was determined by a modified Fiske–Subbarrow method (Bartlett, 1959). Inorganic phosphate was determined by the same method without digestion of the sample. Quantitative ninhydrin reaction was carried out by a modified Moore and Stein procedure (Yokoi, 1959).

Results

Preparation of DIP-Enzymes

The reaction of DFP with enzyme proteins was conducted by either one of the following two methods. *Method 1* was a procedure essentially the same as that described in the preceding communication (Murachi and Yasui, 1965). The enzyme was incubated in 0.25 M Tris buffer at pH 8.2 and at 30° with DFP which was added in portions over a period of 1–5 hr. In *method 2*, a solution of mercuri-stem bromelain was mixed with DFP in a pH-Stat assembly, Radiometer Model SBR2/SBU1/TTT1 Autotitrator, at pH 8.2 and 25° under nitrogen stream. The syringe of the pH-Stat contained 2 N NaOH to maintain the pH at 8.2. The incubation was continued for 3 hr. Amounts of enzyme proteins and DFP used for reactions are shown in Table I. The molar ratio of DFP to the enzyme protein was varied in order to obtain DIP-enzymes with varying degrees of phosphorus incorporation, since the reaction was known to be dependent on the concentration of DFP (Murachi and Yasui, 1965).

In both methods, at the end of the incubation period the reaction mixture was filtered through a column of appropriate size with Sephadex G-25 gel at pH 5.2. The enzyme protein was precipitated with ammonium sulfate and dissolved in water, and the solution was dialyzed *vs.* water according to the procedure described in the preceding communication (Murachi and Yasui, 1965). The yield, phosphorus content, and caseinolytic activity of the DIP-enzymes obtained are summarized in Table I. Samples 7, 10, and 14 were those obtained by method 1 and were employed mainly for the experiments of acid and enzymatic hydrolysis, while samples 48–51, obtained by method 2, were used for spectral studies.

Acid Hydrolysis of DIP-Enzymes

Acid hydrolysis of the DIP-enzymes and column chromatographic analysis for phosphorus compounds were carried out by a procedure essentially the same as that described by Schaffer *et al.* (1953).

A solution of 317 mg of DIP-stem bromelain (sample 10), containing 14.0 μmoles of phosphorus, in 60 ml of 2 N HCl was heated in a sealed tube at 100° for 18 hr. Small aliquots of the hydrolysate were withdrawn for phosphorus analyses and the remainder was concentrated *in vacuo* to dryness. The residue was then dissolved in 5 ml of 0.05 N HCl and the solution was applied to a 0.9 × 110 cm column with Dowex 50 (H^+) (8% cross-linkage, 200–400 mesh) resin which had been equilibrated with 0.05 N HCl. The column was washed with 0.05 N HCl and 2-ml fractions were collected. Each fraction up to fraction 20 was analyzed for in-

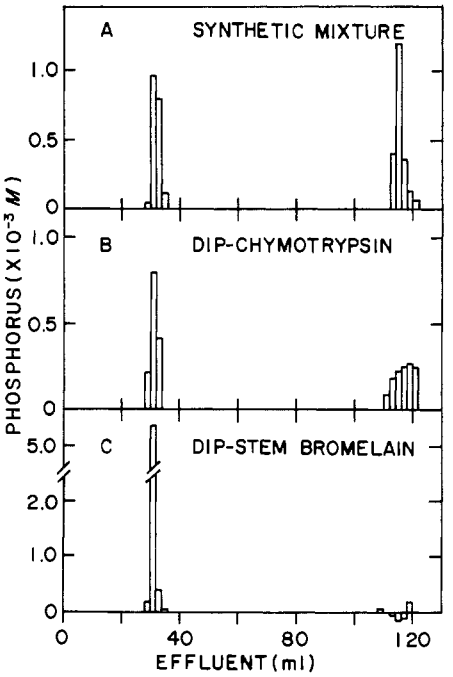


FIGURE 1: Chromatography of phosphorus compounds on Dowex 50 (H^+). A, a synthetic mixture of 5 μmoles of KH_2PO_4 and 5 μmoles of *O*-phosphoryl-DL-serine; B, acid hydrolysate of 190 mg of DIP-chymotrypsin, sample 7; C, acid hydrolysate of 317 mg of DIP-stem bromelain, sample 10. Acid hydrolysis was carried out with 2 N HCl at 100° for 18 hr. The sample was applied to a 0.9 × 110 cm column and the elution was made with 0.05 N HCl; 2-ml fractions were collected. Each fraction up to No. 20 was analyzed for inorganic phosphate and each of the subsequent fractions for organic phosphorus. For the recovery of phosphorus compounds, see Table II.

organic phosphate and each of the subsequent fractions for organic phosphorus.

In the case of DIP-chymotrypsin, 190 mg of sample 7, containing 7.1 μmoles of phosphorus, was hydrolyzed in 42 ml of 2 N HCl, and the hydrolysate was fractionated and analyzed for phosphorus compounds in the same way as above. A control experiment was also carried out with a mixture of *O*-phosphorylserine and inorganic phosphate. Thus, 5 μmoles each of *O*-phosphoryl-DL-serine and KH_2PO_4 was dissolved together in 5 ml of 0.05 N HCl, and the solution was fractionated with a Dowex 50 column as described above. In the last experiment, analyses were also made for ninhydrin color values of the fractions. The elution patterns obtained for phosphorus compounds are shown in Figure 1. The calculated recoveries in inorganic phosphate and organic phosphorus are summarized in Table II. In Table II are also shown the results obtained by Schaffer *et al.* (1953) with ^{32}P-labeled DIP-chymotrypsin.

A comparison of the chromatograms for the samples and for the mixture of authentic compounds (Figure 1) indicates that the first peak corresponds to inorganic phosphate and the second peak to *O*-phosphorylserine. The positions of these peaks relative to the size of the column used were found to be almost the same as those reported by Schaffer *et al.* (1953). When fractions from the synthetic mixture were examined by a quantitative ninhydrin reaction, the obtained pattern exactly corresponded to that of the second peak in Figure 1A with a quantitative recovery in the color value, giving support to the identity of the second peak material with *O*-phosphorylserine. It is apparent, therefore, that the acid hydrolysis of DIP-chymotrypsin under the conditions employed gave both inorganic phosphate and *O*-phosphorylserine, while that of DIP-stem bromelain gave almost exclusively inorganic phosphate.

TABLE II: Recovery of Phosphorus Compounds in Chromatography on Dowex 50 (H^+) with 0.05 N HCl.[a]

Sample Applied	Phosphorus Applied (μmoles)	Phosphorus Applied (%)	Phosphorus Found: Inorganic P in 1st Peak (μmoles)	Phosphorus Found: Inorganic P in 1st Peak (%)	Phosphorus Found: Organic P in 2nd Peak (μmoles)	Phosphorus Found: Organic P in 2nd Peak (%)	Phosphorus Found: Total (%)
Synthetic mixture[b]	10.0	100	4.60	46.0	4.81	48.1	94.1
Acid hydrolysate of DIP-stem bromelain (sample 10)	14.0	100	13.2	94.5	≃0		94.5
Acid hydrolysate of DIP-chymotrypsin (sample 7)	7.10	100	3.13	44.1	2.67	37.6	81.6
Acid hydrolysate of DIP-chymotrypsin (Schaffer *et al.*, 1953)[c]		100		50.5		31.2	81.7

[a] Data were taken from the results shown in Figure 1. [b] The mixture contained 5 μmoles each of *O*-phosphoryl-DL-serine and KH_2PO_4. [c] Per cent recoveries were calculated from radioactivities of the materials.

As shown in Table II, a 37.6% recovery of *O*-phosphorylserine from DIP-chymotrypsin was noted in the present experiment, and this was in good agreement with the reported value (Schaffer *et al.*, 1953). Contrary to this, an almost quantitative recovery of phosphorus as inorganic phosphate from DIP-stem bromelain indicates an acid lability of the phosphoryl linkage that has originally been present in DIP-stem bromelain.

Enzymatic Hydrolysis of DIP-Stem Bromelain

For the purpose of isolating any phosphorylated amino acid from DIP-stem bromelain, an hydrolysis by enzymatic means was the method of choice, since the preceding experiments had demonstrated that the phosphoryl linkage in DIP-stem bromelain was much more acid labile than that in DIP-chymotrypsin. Thus, DIP-stem bromelain was digested by a combined use of proteinases from *Streptomyces griseus* and *Bacillus subtilis*, and the digest was fractionated successively by paper chromatography, paper electrophoresis, and rechromatography on paper as follows.

Digestion of DIP-Stem Bromelain by Proteinases (Step 1). A solution of 340 mg of DIP-stem bromelain (sample 14) in 15 ml of 0.01 M calcium acetate was heated in a boiling water bath for 4 min. The pH of the suspension of the coagulated protein was then adjusted to 8.3 with ammonia, and a small amount of thymol crystals was added as the disinfectant. The digestion was started by adding 30 mg of the partially purified *Streptomyces griseus* proteinase to the suspension, and the mixture was incubated at 37°. On the second day 30 mg of *Bacillus subtilis* proteinase was added to the mixture, and on the third and the following days 10 mg each of alternately one of the two proteinases was added daily until the total sum of the proteinases added amounted to 100 mg. The incubation was then continued for an additional 48 hr. At the end of the incubation period the pH of the hydrolysate was adjusted to 5.2 with 1 N acetic acid and the mixture was heated in a boiling water bath for 7 min. After standing for 1 hr in the cold, the faint turbidity was centrifuged off.

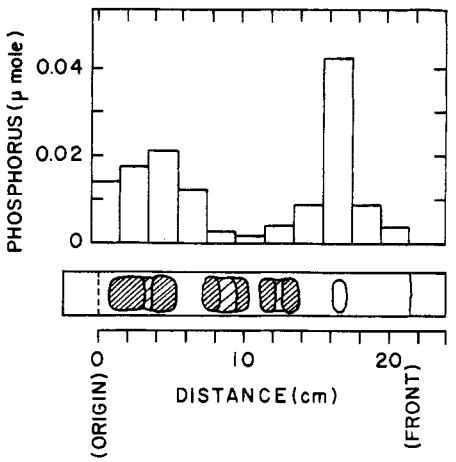


FIGURE 2: Paper chromatography of enzymatic digest of DIP-stem bromelain with 1-butanol–acetic acid–water (4:1:5). Phosphorus contents of 2-cm sections of a guide strip are shown in the upper part of the figure. A tracing of the ninhydrin staining of another guide strip is shown in the lower part. The relative intensities of staining are indicated by the heaviness of cross-hatching. Digestion of 340 mg of DIP-stem bromelain, sample 14, in 15 ml of 0.01 M calcium acetate was carried out with 50 mg of *Streptomyces griseus* proteinase and 50 mg of *Bacillus subtilis* proteinase at pH 8.3 and at 37° for 7 days. A 1.5-ml portion of the deproteinized supernatant was applied to a Toyo No. 50 paper (40 × 40 cm).

Paper Chromatography of Enzymatic Digest (Step 2). A 1.5-ml portion of the enzymatic digest obtained above was applied to a 40 × 40 cm Toyo No. 50 filter paper as a streak and the paper chromatograph was developed for 20 hr with 1-butanol–acetic acid–water (4:1:5). A guide strip, 2 cm in width, was cut out from the sheet and stained with ninhydrin. It was found that a faintly purple spot with an R_F value of 0.77 appeared far ahead of heavily stained spots. Another guide strip,

TABLE III: Analyses of a Phosphorus-Containing Amino Acid Isolated from the Enzymatic Digest of DIP-Stem Bromelain.[a]

Sample	Organic P (μmoles/ml)	Inorganic P (μmoles/ml)	Amino Group as Leucine Equivalent (μmoles/ml)	Ratio (Amino Group/P)
Before acid hydrolysis	3.20	0.00	2.94	0.92
After acid hydrolysis	—	3.15	2.77	0.88

[a] DIP-stem bromelain, sample 14, was used as the starting material. Analyses for phosphorus content and ninhydrin color value were made with 0.02-ml aliquots of the sample solution before acid hydrolysis. A 0.25-ml aliquot was mixed with 0.25 ml of 4 N HCl in a sealed tube and the hydrolysis was carried out at 100° for 19 hr; 0.05-ml each of the hydrolysate was used for the determination of inorganic phosphate and of ninhydrin color value. The data obtained are expressed as μmoles/ml of the original sample solution.

TABLE IV: Characterization by Paper Chromatography and Paper Electrophoresis of a Phosphorus-Containing Amino Acid Isolated from the Enzymatic Digest of DIP-Stem Bromelain.[a]

Compound	R_F Value on Paper Chromatogram[b]			Electrophoretic Mobility on Paper[c] (cm)
	Solvent 1	Solvent 2	Solvent 3	
Sample before acid hydrolysis	0.79	0.64	0.80	+4.8
Sample after acid hydrolysis	0.39	0.25	0.58	+7.0
O-DIP-L-tyrosine	0.79	0.65	0.80	+4.8
L-Tyrosine	0.38	0.26	0.58	+7.0

[a] DIP-stem bromelain, sample 14, was used as the starting material. The dried residue in step 4 (see text) was examined before and after a hydrolysis in 2 N HCl at 100° for 19 hr, in comparison with authentic compounds. [b] Solvent systems for paper chromatography: 1, 1-butanol–acetic acid–water (4:1:5); 2, ethanol–ammonia–water (18:1:1); 3, 1-butanol–pyridine–water (1:1:1). Toyo No. 50 filter paper was used. [c] At pH 2.3, formic acid–pyridine–water (50:3:950), at 3000 v for 25 min. Toyo No. 50 paper was used.

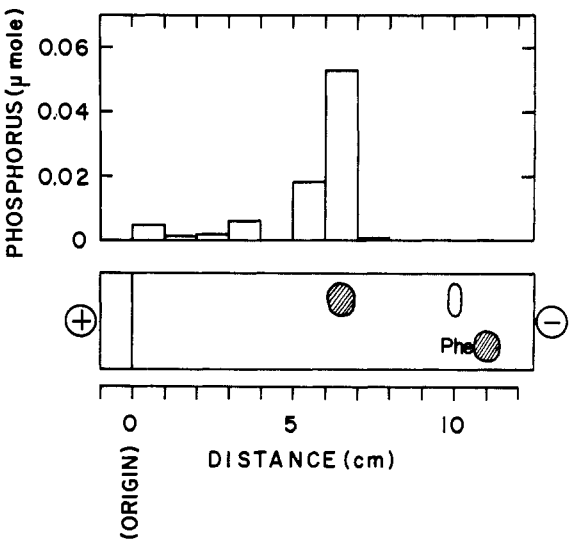


FIGURE 3: High voltage electrophoresis on paper of the R_F 0.77 material obtained in step 2 (see text). Phosphorus contents of 1-cm sections of a guide strip are shown in the upper part of the figure. A tracing of the ninhydrin staining of another guide strip is shown in the lower part; Phe, phenylalanine as reference. Electrophoresis was carried out on Toyo No. 50 paper (40 × 10 cm) at pH 2.3 (formic acid–pyridine–water, 50:3:950), for 25 min at 3000 v.

4 cm in width, was then made, and this was cut into 2-cm sections parallel to the solvent front. Each section was eluted with 3 ml of water and the eluate was analyzed for the phosphorus content. The results of the analysis are shown in Figure 2, together with a tracing of the ninhydrin staining. As shown in Figure 2, approximately 50% of the organic phosphorus present in the enzymatic digest was found to be associated with the R_F 0.77 material. This material was located on the unstained paper by the aid of the guide strips described above and eluted with water. Further lots of the material were obtained by carrying out chromatography with five more sheets of filter paper. The eluates were combined and concentrated *in vacuo* to dryness, and the residue was dissolved in 1.0 ml of water.

Purification of the Phosphorus-Containing Amino Acid by Paper Electrophoresis (Step 3). A 0.3-ml portion of the step 2 material was applied to a 40 × 10 cm Toyo No. 50 filter paper as a 6-cm streak. High voltage electrophoresis was carried out at pH 2.3 (formic acid–pyridine–water, 50:3:950) and at 3000 v for 25 min. With guide strips of appropriate sizes, ninhydrin staining and phosphorus analysis were conducted in the same way as described for step 2. The results obtained are illustrated in Figure 3. As shown in the figure more than 80% of the phosphorus is found in association with a ninhydrin-positive material which moves toward the anode more slowly than common neutral amino acids. From the unstained paper this material was eluted with water. Additional batches of the material were obtained from two more sheets of filter paper treated in the same way. The combined eluates were evaporated *in vacuo* to dryness, and the residue was dissolved in 1.0 ml of water.

Rechromatography on Paper (Step 4). The step 3 material was further purified by preparative paper chromatography with a solvent system of 1-butanol–acetic acid–water (4:1:5). Ninhydrin staining of the developed guide strip revealed one major spot with R_F 0.79. The material corresponding to this spot was eluted with water from the unstained filter paper and the eluate was concentrated *in vacuo* to dryness. The recovery of the organic phosphorus from the enzymatic digest up to the end of step 4 was found to be approximately 32%.

Identification of O-DIP-L-Tyrosine. The dried residue in step 4 was dissolved in 1.0 ml of water and with

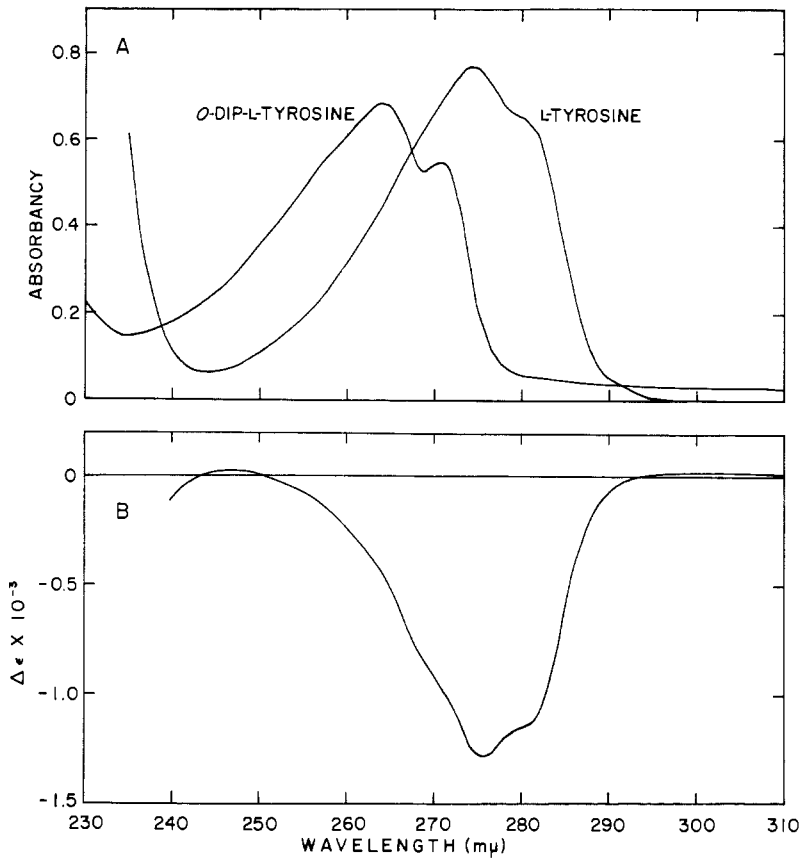


FIGURE 4: Ultraviolet absorption spectra of L-tyrosine and *O*-DIP-L-tyrosine (A), and difference spectra of *O*-DIP-L-tyrosine *vs.* L-tyrosine (B). Measurements of spectra were made with 0.5491×10^{-3} M-L-tyrosine in 0.05 M sodium acetate buffer at pH 5.2 and with 1.834×10^{-3} M *O*-DIP-L-tyrosine in the same buffer. In section B is shown a plot of the calculated difference in molar absorbancy between the two compounds.

0.02-ml aliquots of the solution analyses were made for the phosphorus content and the ninhydrin color value. The analyses were also carried out after an acid hydrolysis of the sample in 2 N HCl at 100° for 19 hr. The results of these analyses are shown in Table III, indicating the presence of 1 mole of organic phosphorus per mole of amino group in the original sample which can be hydrolyzed to give 1 mole of inorganic phosphate. Paper chromatography of the acid hydrolysate of the sample revealed the presence of a single spot with ninhydrin staining. Identification of the ninhydrin-positive compounds before and after the acid hydrolysis was made by means of paper chromatography and paper electrophoresis. Table IV summarizes the results obtained for these samples in comparison with those for authentic *O*-DIP-L-tyrosine and L-tyrosine. Excellent agreement is noted between values for the samples and those for the authentic compounds. Although no study was made on the rotatory properties of the samples, it must be reasonable to assume that the amino acids concerned were of the L configuration since they were derived from the protein by enzymatic hydrolysis. It was thus concluded that a substantial amount of *O*-DIP-L-tyrosine was obtained from an enzymatic hydrolysate of DIP-stem bromelain.

Spectral Studies of DIP-Stem Bromelain

Difference Spectra of O-DIP-L-Tyrosine vs. L-Tyrosine. Figure 4A shows the ultraviolet absorption spectra of L-tyrosine and *O*-DIP-L-tyrosine in 0.05 M sodium acetate buffer at pH 5.2. The latter compound was the specimen synthesized in this laboratory. *O*-DIP-L-tyrosine shows two absorption maxima with molar absorbancies $\epsilon_{264m\mu}$ 373 and $\epsilon_{271m\mu}$ 298. Simpson *et al.* (1963) reported that *N,O*-diacetyltyrosine absorbs maximally at 262 mμ with $\epsilon_{262m\mu}$ 620. In Figure 4B is shown the difference in molar absorbancy of *O*-DIP-L-tyrosine *vs.* L-tyrosine at pH 5.2. The maximal change in difference spectra is noted at 276 mμ with $\Delta\epsilon_{276m\mu}$ −1280. A value of $\Delta\epsilon_{278m\mu}$ −1160 was reported by Simpson *et al.* (1963) for the difference spectra of *N,O*-diacetyltyrosine *vs.* tyrosine at pH 7.5.

Difference Spectra of DIP-Stem Bromelain vs. Stem Bromelain. The protein concentration of the samples of DIP-stem bromelain (No. 49–51) and the reference enzyme preparation (No. 48) were determined by the biuret reaction. Since molar concentration of the reference enzyme solution had been known by measuring absorbancy at 280 mμ, the biuret color values obtained for DIP-enzymes could also be related to their molar concentrations on the assumption that no pep-

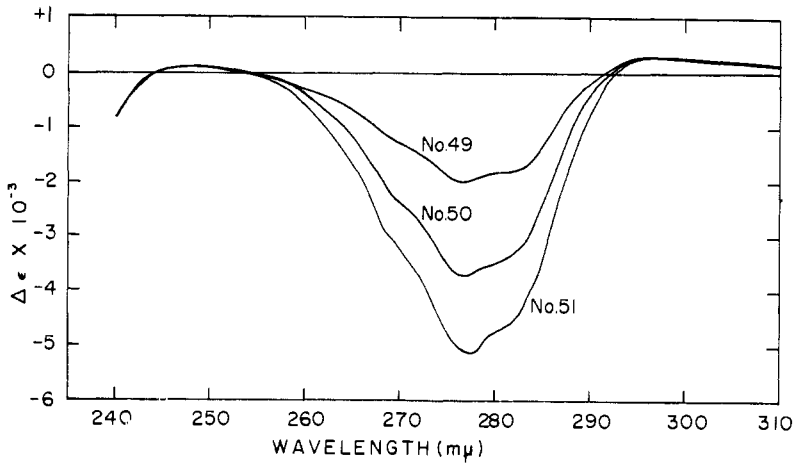


FIGURE 5: Difference spectra of DIP-stem bromelain *vs.* stem bromelain. Samples of DIP-stem bromelain (No. 49, 50, and 51) were dissolved in 0.05 M sodium acetate buffer at pH 5.2 to make a 2.3×10^{-5} M solution. Light absorption of the sample was measured against 2.3×10^{-5} M solution of the reference enzyme preparation (sample 48) in the same buffer.

tide cleavage had occurred during the course of preparing these samples. The molar absorbancies at 280 mμ thus calculated for samples 49, 50, and 51 were 6.49×10^4, 6.31×10^4, and 6.18×10^4, respectively. It was then possible to dilute the sample and the reference enzyme solutions with 0.05 M sodium acetate buffer to make an identical protein concentration, *i.e.*, 2.3×10^{-5} M, at which concentration spectrophotometric determinations were carried out. The difference spectra thus obtained for three different DIP-enzymes *vs.* the reference enzyme preparation are shown in Figure 5. Each one of the three spectra shown in the figure markedly resemble the difference spectra of *O*-DIP-L-tyrosine *vs.* L-tyrosine (Figure 4B), with the maximal change in spectra at 277 mμ. This fact strongly suggests diisopropylphosphorylation of a certain number of tyrosine hydroxyl groups of the enzyme protein. The number of such groups phosphorylated per molecule can be calculated by dividing the observed difference in molar absorbancy at 277 mμ for proteins by $\Delta\epsilon_{277m\mu}$ of DIP-L-tyrosine *vs.* L-tyrosine. A $\Delta\epsilon_{277m\mu}$ of −1250 with the latter compounds (Figure 4B) was used for calculation, and the values obtained are shown in the fourth column of Table V. Good agreement is noted between these values and the moles of phosphorus/mole of enzyme protein previously known for these samples (see the third column of Table V).

Absorbancy at 295 mμ of DIP-Stem Bromelain in Alkaline Medium. The number of ionizable tyrosine hydroxyl groups of three different samples of DIP-stem bromelain (No. 49–51) and the reference enzyme preparation (No. 48) were determined by a spectrophotometric method. To 4.5 ml of an approximately 2×10^{-5} M solution of the test protein was added either 0.5 ml of 0.1 M sodium acetate buffer at pH 5.2 or 0.5 ml of 0.1 N NaOH, and the mixture was allowed to stand at 17° for exactly 10 min. The absorbancy at 295 mμ of the alkaline solution was then read against the solution at pH 5.2. The difference in absorbancy at 295 mμ was found to increase rapidly during the first 5 min after the solution was made alkaline, reflecting a denaturing process of unfolding of the enzyme protein.[4] After 10 min such a process comes closer to the end, permitting more exact measurements of absorbancy with a manually operated instrument. In a separate experiment it was found that *O*-phosphoryl linkage in *O*-DIP-L-tyrosine remained practically intact by an incubation in 0.01 N NaOH for 10 min at 17°. Ashbolt and Rydon (1957) reported the first-order kinetics of the hydrolysis of *O*-DIP-L-tyrosine in 1 N NaOH at 37° with a calculated rate constant of 0.0046 min^{-1}. The latter value corresponds to 4.4% hydrolysis of the phosphoryl linkage after 10 min, but this was the result obtained with 100 times as high concentration of alkali as that in the present experiment.

From the observed difference in absorbancy at 295 mμ of the reference preparation (No. 48) in 0.01 N NaOH *vs.* at pH 5.2, a difference in molar absorbancy, $\Delta\epsilon_{295m\mu}$, was calculated to be 2.85×10^4. This corresponds to an ionization of 12.28 phenolic groups per molecule, assuming that $\Delta\epsilon_{295m\mu}$ of tyrosine before and after ionization of the phenolic group is 2330, as was observed in this laboratory. A value, $\Delta\epsilon_{295m\mu}$ 2300 for tyrosine, was reported by Crammer and Neuberger (1943). Using 2330 for $\Delta\epsilon_{295m\mu}$ of tyrosine, numbers of ionizable phenolic groups per molecule were calculated also for DIP-enzymes. Thus, samples 49, 50, and 51 were found to have 10.73, 9.55, and 8.48 phenolic groups per molecule, respectively, which are ionized in 0.01 N NaOH. These data are presented in the fifth column of Table V as a decrease in the number of ionizable phenolic groups of each DIP-enzyme as compared to the reference preparation. The value for such decrease was

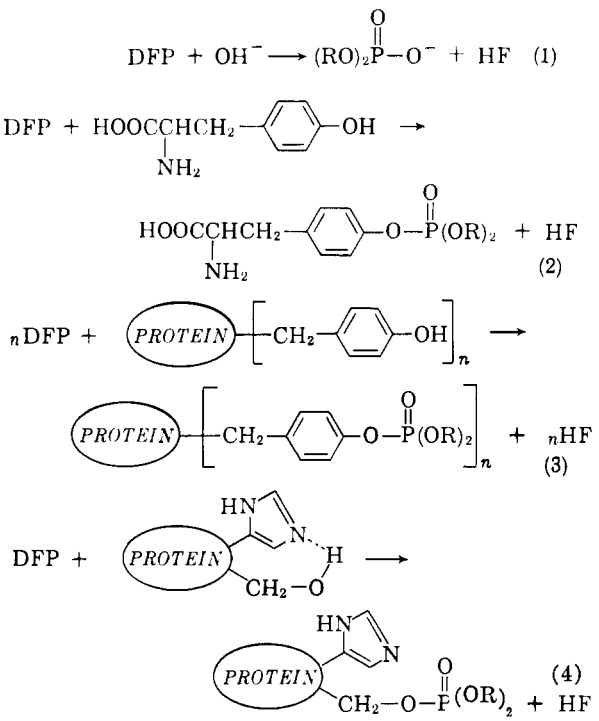
[4] A. Tachibana and T. Murachi, unpublished observation.

expected to agree with the number of tyrosyl residues modified that had been determined, as described above, by a measurement of difference spectra of *O*-DIP-enzyme *vs.* the reference preparation. In fact, a satisfactorily good agreement is noted between the data in the fourth and the fifth columns of Table V.

Discussion

The experimental data described above clearly indicate that the phosphorylation by DFP of stem bromelain occurred at the hydroxyl groups not of seryl but of tyrosyl residues of the enzyme protein. Four major pieces of evidence are as follows. First, the absence of *O*-phosphorylserine in the acid hydrolysate of DIP-stem bromelain (Figure 1 and Table II) has lent support to the nonreactivity of seryl residues of stem bromelain with DFP. An almost quantitative recovery of phosphorus as inorganic phosphate (Table II) has indicated that the phosphoryl linkage in DIP-stem bromelain is more acid labile than that of *O*-phosphorylserine. Second, the occurrence of alkylphosphorylation of tyrosine hydroxyl groups has been positively evidenced by the isolation of *O*-DIP-L-tyrosine from the enzymatic hydrolysate of DIP-stem bromelain. *O*-Phosphoryl linkage in *O*-DIP-L-tyrosine was reported to be hydrolyzed by heating the solution in 1 N sulfuric acid at 100° (Ashbolt and Rydon, 1957). From the calculated first-order rate constant, 0.0016 min^{-1}, the half-life of the compound was known to be approximately 8 hr. In view of this, the acid hydrolysis employed in the present experiment, *i.e.*, heating DIP-stem bromelain in 2 N HCl at 100° for 18 hr, seems to be sufficient to cause an extensive cleavage of the phosphoryl linkage to yield almost quantitatively inorganic phosphate (Table II). Third, alkylphosphorylation of the tyrosine hydroxyl groups of stem bromelain has also been proved by the difference spectra of DIP-enzyme *vs.* the reference enzyme preparation (Figure 5). The pattern of spectra obtained is almost identical with that of *O*-DIP-L-tyrosine *vs.* L-tyrosine (Figure 4). Fourth, it has been demonstrated that the number of moles of phosphorus incorporated into stem bromelain after the latter was allowed to react with DFP is in good agreement with the number of tyrosyl residues modified per molecule of enzyme protein which was determined by two independent measurements of ultraviolet absorption (Table V). The one is pertaining to a direct measurement of difference spectra of DIP-stem bromelain *vs.* the reference enzyme preparation, while the other is related to indirect determination from the number of ionizable phenolic groups before and after the reaction with DFP. Had any major phosphoryl moiety other than those attached to the tyrosyl residues been present in the molecule, significant discrepancies would have been observed between the phosphorus content and both of the spectral data. It is thus concluded that the phosphorylation of stem bromelain has occurred exclusively at the phenolic hydroxyl groups of tyrosyl residues of the enzyme protein.

The alkylphosphorylation of tyrosyl residues of stem bromelain is not an unexpected phenomenon in view of the known reactivity of the phenolic hydroxyl group of tyrosine with DFP (Ashbolt and Rydon, 1957). The phenomenon is also not incompatible, in principle, with the well-known fact that a single seryl residue is alkylphosphorylated by DFP in cases of chymotrypsin and some other hydrolases (Hartley, 1960). In all instances the basic principle of the reaction is one, *i.e.*, a displacement of fluorine of DFP by a nucleophilic atom of the reactant (Heath, 1961). Four types of nucleophiles are now known to react with DFP to form P–O bonds.



where R stands for diisopropyl group.

Equation 1 is the hydrolysis of DFP in an alkaline medium, and the rate of the reaction was found to increase proportionately with the concentration of hydroxide ion (Murachi and Yasui, 1965). Reaction 2 represents the alkylphosphorylation of the phenolic hydroxyl group of free tyrosine which was reported by Ashbolt and Rydon (1957). Reaction 3 is what we have demonstrated to occur with stem bromelain. The same type of reaction occurs also with egg white lysozyme (Murachi and Inagami, 1963). Jandorff *et al.* (1955) claimed that tyrosine was involved in a slow further reaction of DFP with chymotrypsin, subsequent to the initial fast inactivation, and other nonenzyme proteins, but their report seems to be lacking in spectral data to support this claim. Reaction 2 was reported to proceed much more slowly compared to the inactivation reaction with DFP of some esterases (Ashbolt and Rydon, 1957). Since reaction 3 is a reaction analogous to reaction 2, it is quite natural to observe that the rate of phosphorylation of stem bromelain by DFP was very slow (Murachi and Yasui, 1965). Similarly, an essentially nonspecific nature of reaction 3 is consistent with

TABLE V: Alkylphosphorylation of Tyrosyl Residues of Stem Bromelain by DFP.

Sample	DFP Added[a] (moles/mole enzyme)	Phosphorus Found[a] (moles/mole enzyme)	Number of Tyrosyl Residues Modified/Molecule	
			Calculated from $\Delta\epsilon_{277m\mu}$[b]	Calculated from $\Delta\epsilon_{295m\mu}$[c]
48	0	0.00	—	—
49	77.4	1.49	1.58	1.55
50	193	2.97	2.96	2.73
51	387	3.87	4.06	3.80

[a] Values in both of these columns are taken from Table I. [b] The values were obtained from difference spectra of samples (No. 49–51) *vs.* the reference preparation (No. 48) at pH 5.2. Patterns of difference spectra are shown in Figure 5. For details of calculation, see text. [c] The values are the difference in number of ionizable phenolic groups between DIP-enzymes and the reference preparation, as calculated from $\Delta\epsilon_{295m\mu}$ of each preparation in 0.01 N NaOH *vs.* at pH 5.2. For details of calculation, see text.

the finding that several tyrosyl residues per molecule of enzyme protein can be phosphorylated (Table I; Murachi and Yasui, 1965). Stem bromelain contains 19 tyrosyl residues per molecule (Murachi, 1964a), of which 7 to 9 are supposed to be "exposed" to the outside surface of the enzyme protein.[5] At pH 8.2, reaction 3 is theoretically possible to occur equally well with any one of these accessible tyrosyl residues unless some of them are postulated to be more reactive than the others. No positive evidence has been made available to indicate that one tyrosyl residue or two of a stem bromelain molecule is more reactive than the other tyrosyl residues, although this was reported to be true in the case of carboxypeptidase A (Simpson *et al.*, 1963). Therefore, the number of tyrosyl residues phosphorylated per molecule shown in Table I does not always need to be an integer, but it may represent an average of the molecules with varying degrees of phosphorylation. It is still open to question, however, how widely the degrees of phosphorylation of individual molecules are distributed around the mean value observed.

Reaction 4 is the most commonly known one which proceeds very rapidly and involves a single specific seryl residue to lead to an inactivation of the enzyme. The reaction is believed to be a result of a concerted action of seryl and histidyl residues in the active site of the enzyme (Desnuelle, 1960) and is written above with a hydrogen bonding of the serine hydroxyl group to one of the imidazoles (Hartley, 1964). Such a hydrogen bonding may increase the nucleophilicity of the oxygen atom of the serine hydroxyl group so that it reacts preferentially with a phosphorylating agent like DFP or an acylating agent like *p*-nitrophenyl acetate (Oosterbaan and van Adrichem, 1958); otherwise a serine hydroxyl group could have hardly been the site of these reactions since ordinarily a serine hydroxyl oxygen is less nucleophilic than a phenolic hydroxyl oxygen, reflecting a higher p*K* value of the primary alcoholic group. Again in view of the nucleophilicity of the side chain group, it is quite reasonable to find out that tyrosine was the only one of the nine amino acids studied, including serine, which reacted with DFP at pH 7.8 (Ashbolt and Rydon, 1957). Stem bromelain is one of the thiol proteinases (Murachi and Neurath, 1960) and has not been found to contain any reactive seryl residue in its active site. The active SH group of a cysteinyl residue has been found not to be involved in the alkylphosphorylation by DFP (Murachi and Yasui, 1965). It is not surprising, therefore, to find that only phenolic hydroxyl groups of tyrosyl residues were the sites of alkylphosphorylation of this enzyme.

It is interesting to note in Table I that a DIP-enzyme like sample 50 or 51, which had been rather extensively alkylphosphorylated at its tyrosyl residues, showed an almost unchanged specific activity toward casein as compared to the reference enzyme preparation. However, when the activities of these DIP-enzymes were studied with synthetic substrates, it was found that the alkylphosphorylation had caused an alteration in substrate specificity (Murachi, 1964b), suggesting that some role in the catalysis is played by tyrosyl residue or residues of the enzyme protein. The functional tyrosyl residues of carboxypeptidase A have been reported (Simpson *et al.*, 1963). Details of a specificity experiment with DIP-stem bromelain will be published elsewhere.

Acknowledgments

We wish to thank Professor H. N. Rydon of the University of Exeter, Exeter, England, for his generosity in supplying us with a specimen of *O*-DIP-L-tyrosine. Our thanks are also due to Miss Tokiko Miyake for her excellent assistance in much of this work.

[5] A. Tachibana and T. Murachi, unpublished observation.

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Syntheses of Deoxynucleoside 3′-Triphosphates*

John Josse and J. G. Moffatt

ABSTRACT: Efficient chemical syntheses of the 3′-triphosphates derived from the four commonly occurring deoxyribonucleosides are described. The syntheses involve conversion of the chemically synthesized or enzymatically isolated deoxynucleoside 3′-phosphates into the 3′-phosphoromorpholidates, which are then condensed with tributylammonium pyrophosphate in anhydrous dimethyl sulfoxide.

Isolated yields of 52–72% of the pure triphosphates were obtained by ion-exchange chromatography, and the structures were confirmed both chemically and enzymatically.

The findings of Canellakis *et al.* (1965) that extracts of *Bacillus subtilis* catalyze phosphorylation of thymidine 3′-diphosphate (3′-TDP)[1] to the corresponding triphosphate and of Josse (unpublished) that kinases specific for deoxynucleoside 3′-monophosphates can be purified from extracts of *Escherichia coli* have raised the question of what role, if any, such deoxynucleoside 3′-triphosphates might play in the cell. (A recent report by Coutsogeorgopoulos and co-workers (1965) has documented formation of 3′-TTP by extracts of regenerating rat liver.) In an effort to investigate this question, which could conceivably have important

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[1] Abbreviations used are: 3′-dAMP, 3′-dGMP, 3′-dCMP, 5-Me-3′-dCMP, and 3′-TMP: the deoxynucleoside 3′-monophosphates, respectively, of adenine, guanine, cytosine, 5-methylcytosine, and thymine; 3′-d( )M: deoxynucleoside 3′-phosphoromorpholidates of the various bases; 3′-d( )DP and 3′-d( )TP: deoxynucleoside 3′-di- and triphosphates, respectively, of the various bases; α^{32}P-3′-TDP and α^{32}P-3′-TTP: thymidine 3′-di- and triphosphate, respectively, labeled with ^{32}P in the α-phosphate (that esterified to sugar).