from phosphoprotein and not from some other source. Since it has been shown (McIndoe, 1959) that no further quantities of alkali-labile phosphorus are released from the plasma phosphoproteins after incubation for 18 hr. it is considered that this method of determination of phosphoprotein phosphorus is likely to yield an accurate value for the total quantities present.

Experiments such as the above, though establishing the nature of part at least of the protein-bound phosphorus, do not yield information about the overall nature of the phosphoprotein. Tentative evidence has been presented suggesting that phosvitin may be the only phosphoprotein present, part combining with lipid to form a precipitate on dilution of the plasma and part remaining in solution (McIndoe, 1959; Mok *et al.* 1961). Such a suggestion offers a simple explanation for the different composition of the lipophosphoprotein complexes obtained with different anticoagulants during the collection of blood (Table 1), and for the similar specific radioactivities of the phosphoprotein phosphorus derived from the two fractions. However, the accuracy of the suggestion can be decided only by isolation and further study of the phosphoproteins.

SUMMARY

1. Phosphorylserine has been isolated and identified as the sole phosphorylated amino acid present in acid hydrolysates of the plasma phosphoproteins of the laying hen. No phosphorylserine was detected in hydrolysates of plasma from non-laying hens or from cockerels.

2. After the injection of radioactive phosphate the specific radioactivity of phosphorylserine, isolated after different times from two plasma-phosphoprotein fractions, was the same in both fractions and similar to that of inorganic phosphate released on incubating the phosphoproteins with dilute alkali.

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The Influence of Adjacent Phosphate and Hydroxyl Groups on Amino Acid Esters

By ZOE A. SHABAROVA, N. A. HUGHES AND J. BADDILEY

Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne

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Perhaps the most remarkable property of teichoic acids is the considerable reactivity of their D-alanine ester residues. Teichoic acids from bacterial walls or from intracellular material all possess these reactive ester groups and, although their

number varies somewhat in samples prepared from different organisms and by different techniques, values as high as 0.75 have been reported for the ratio alanine:P in the polymers.

The glycerol teichoic acids are polymers of

glycerol phosphate in which glycerol residues are joined together through phosphodiester groups at the 1 and 3 positions (cf. Kelemen & Baddiley, 1961), although in some cases sugars or amino sugars may be attached glycosidically to some of the glycerol residues (unpublished observations by A. J. Wicken, P. Critchley, A. L. Davison and D. C. Ellwood). In the glycerol teichoic acids that contain only small amounts of sugar or from which sugar is absent, the alanine is in ester linkage with the 2-hydroxyl groups of the glycerol residues.

The location of the alanine ester groups in ribitol teichoic acids has been examined so far in three cases. In those from the walls of *Bacillus subtilis*, *Staphylococcus aureus* and *Lactobacillus arabinosus*, these ester groups are apparently attached to hydroxyl at either the 2- or 3-position on ribitol residues (Armstrong, Baddiley & Buchanan, 1961; Baddiley *et al.* 1961; A. R. Archibald, unpublished observations). It is reasonable to assume that alanine in other ribitol teichoic acids will be located similarly.

The rate of reaction at neutral pH values between hydroxylamine and the alanine ester groups in several teichoic acids has been used previously as a measure of their reactivity (Baddiley & Neuhaus, 1960; Kelemen & Baddiley, 1961); however, qualitative and semi-quantitative experiments with ammonia and alkaline buffers on many teichoic acids support the general conclusion that the alanine ester linkages are unusually reactive (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Archibald, Armstrong, Baddiley & Hay, 1961). The rate of their reaction with hydroxylamine has been compared with that reported for the amino acid ester residues in the nucleic acid-amino acid complexes which are concerned in protein biosynthesis (Berg & Ofengand, 1958). These rates are similar and are considerably higher than those for simple amino acid esters.

Previously, the high reactivity of the nucleic acid derivatives was attributed to the presence of a neighbouring hydroxyl group adjacent to the ester linkage, but studies with model compounds indi-

cate that this is only one of several possible factors (Wieland, Merz & Pfeleiderer, 1960; Zachau, 1960; Zachau & Karau, 1960). A similar explanation is obviously not applicable to the glycerol teichoic acids, though it could apply to the ribitol teichoic acids. A common feature of all teichoic acids and nucleic acids is the presence of phosphodiester linkages; it was thought that, at least in the teichoic acids, the high reactivity of the ester linkages must in some way be concerned with the presence of these phosphate groups. To this end some simple model compounds, based on ethylene glycol, have been synthesized and the reactivity of their ester linkages has been examined.

EXPERIMENTAL

Chromatography. Ascending chromatography was carried out on Whatman no. 1 paper. The following solvent systems were used: A, butan-1-ol-acetic acid-water (4:1:1, by vol.); B, butan-1-ol-acetic acid-water (5:2:3, by vol.); C, propan-1-ol-ammonia (sp.gr. 0.88)-water (6:3:1, by vol.). Paper electrophoresis was performed on Whatman no. 1 paper soaked in 0.1M-sodium acetate (pH 6.0) for 2 hr. with a gradient of 10 v/cm. R_F values are given in Table 1.

Detection of compounds. Amino acids were detected with ninhydrin (Consden & Gordon, 1948). Ethylene glycol was detected with sodium periodate-Schiff reagents (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956). Inorganic and organic phosphates were detected with perchloric acid-ammonium molybdate (Hanes & Isherwood, 1949). Hydroxamates were detected with ferric chloride (Stadtman & Barker, 1950).

Hydrolysis conditions. Acid hydrolyses were carried out in 2N-hydrochloric acid for 3 hr. at 100°. Alkali hydrolyses were performed in 0.1N-sodium hydroxide for 5 hr. at 37°. Incubations with alkaline phosphatase (0.2 mg.) (Sigma Chemical Co.) were carried out in 0.1M-ammonium acetate buffer (1 ml., pH 9.3) for 24 hr. at 37°.

Determination of hydrolysis products. Inorganic phosphate was determined by the method of Chen, Toribara & Warner (1956). Total phosphate was similarly determined after hydrolysis with sulphuric acid-perchloric acid (Hanes & Isherwood, 1949). Alanine was determined by the method of Rosen (1957) and ethylene glycol by the method of Hanahan & Olley (1958). Hydroxamates were

Table 1. *Paper chromatography and electrophoresis of esters*

	R_F in solvents			M^*
	A	B	C	
<i>N</i> -Benzyloxycarbonylalanine hydroxamate	0.95	—	—	—
Alanine hydroxamate	0.35	—	—	—
Alanine amide	—	—	0.59	—
Alanine	—	0.42	0.48	+2.3
2-DL-Alanyloxyethanol	—	0.52	—	+8.3
2-DL-Alanyloxyethyl dihydrogen phosphate	—	0.33	—	+1.0
2-Hydroxyethyl dihydrogen phosphate	—	0.34	0.24	-4.7
Ethylene 1,2-phosphate	—	0.31	0.50	—

* Distance (cm.) moved by compound towards cathode.

prepared and estimated by the methods adopted previously (Kelemen & Baddiley, 1961).

Phosphates of ethylene glycol. Samples of 2-hydroxyethyl dihydrogen phosphate and the cyclic ethylene 1,2-phosphate made by the method of Kumamoto, Cox & Westheimer (1956) were given by Dr J. G. Buchanan.

2-(N-Benzoyloxycarbonyl-DL-alanyloxy)ethanol. *N*-Benzoyloxycarbonyl-DL-alanine (4.46 g., 20 m-moles) and sodium hydroxide (0.08 g., 2 m-moles) were added to a solution of ethylene oxide (15 ml.) in water (50 ml.). The mixture was shaken for 8 hr. at 18°, when no solid remained. Excess of ethylene oxide was removed *in vacuo* and the solution was extracted with ether (3 × 25 ml.). The extract was washed with sodium carbonate solution and water and dried over sodium sulphate. Removal of the ether *in vacuo* left a residue which slowly crystallized. Recrystallization from ethanol-water gave 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)-ethanol (3.00 g., 11 m-moles), as colourless prisms with m.p. 57–58° (Found: C, 58.3; H, 7.0. $C_{13}H_{17}NO_6$ requires C, 58.4; H, 6.6%).

2-DL-Alanyloxyethanol. 2-(*N*-Benzoyloxycarbonyl-DL-alanyloxy)ethanol (0.53 g., 2.0 m-moles) in dioxan (20 ml.) was hydrogenated at 18° and atmospheric pressure with 50% palladium on charcoal (100 mg.), prepared by Linstead & Thomas's method (1940). The apparatus contained an adsorbent for carbon dioxide, and the hydrogen uptake indicated that the reaction was complete in 20 hr. The catalyst was removed and the solution concentrated under reduced pressure to a syrup. This was dissolved in ethanol (10 ml.) and oxalic acid (0.3 g., 3.3 m-moles) in ethanol (10 ml.) was added. Dry ether (50 ml.) was added and the crystalline precipitate was filtered off and recrystallized from ethanol. The acid oxalate (0.30 g., 1.3 m-moles) formed colourless needles, m.p. 123–124° (Found: C, 38.3; H, 5.8. $C_8H_{11}NO_3 \cdot C_2H_2O_4$ requires C, 37.7; H, 5.8%).

2-DL-Alanyloxyethyl dihydrogen phosphate. Dibenzyl phosphorochloridate [from dibenzyl phosphite (1.30 g., 5.0 m-moles) prepared by the method of Atherton, Openshaw & Todd (1945)] was added rapidly to a solution of 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)ethanol (1.30 g., 5.0 m-moles) in dry pyridine (10 ml.) and dry carbon tetrachloride (10 ml.) which had been cooled in an acetone–solid carbon dioxide bath. The solution was kept at –30° for 18 hr. and at room temperature for a further 24 hr. Solid was removed by filtration and the solution was evaporated *in vacuo* at 30°. The syrupy residue was dissolved in carbon tetrachloride (20 ml.) and washed with 2*N*-hydrochloric acid, saturated sodium hydrogen carbonate solution and water, and finally dried over sodium sulphate. Evaporation of solvent gave a gum (1.88 g.), which was dissolved in benzene (20 ml.) and applied to a column (3 cm. × 25 cm.) of silica (100 g., Hopkin and Williams Ltd., M.F.C. grade). Elution with benzene (170 ml.) and ether–benzene (1:9, v/v; 170 ml.) removed some impurities (0.02 g.), and elution with ether–benzene (1:1, v/v; 240 ml.) gave 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)ethyl dibenzyl phosphate (0.7 g.). Further elution with ether–benzene (1:1, v/v; 240 ml.) gave 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)ethanol (0.55 g.). Complete separation of components was achieved.

A sample (200 mg.) of the dibenzyl phosphate ester was hydrogenated in 90% acetic acid (20 ml.) at room temperature and atmospheric pressure with 50% palladium–charcoal (50 mg.); reaction was complete in 20 min. The

catalyst was removed by filtration and the clear solution was poured into acetone (200 ml.). The resulting precipitate (40 mg.) was collected by centrifuging, washed with acetone and dried. This crude product was dissolved in 50% acetic acid (15 ml.), cooled to 0°, and decolorized with charcoal. Acetone was added until the solution became cloudy and the solution was kept at 0°. 2-DL-Alanyloxyethyl dihydrogen phosphate (20 mg., 0.1 m-mole) formed colourless needles, m.p. 235–236° (decomp.) (Found: C, 26.9; H, 6.5. $C_5H_{12}NO_6P \cdot \frac{1}{2}H_2O$ requires C, 27.0; H, 5.8%).

RESULTS AND DISCUSSION

Reaction of *N*-benzyloxycarbonyl-DL-alanine with ethylene oxide in the presence of a catalytic quantity of sodium hydroxide gave 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)ethanol (I) by the general method of Fraenkel-Conrat & Olcott (1944) for the preparation of ethylene glycol monoesters. The benzyloxycarbonyl group was readily removed by hydrogenolysis to give syrupy 2-DL-alanyloxyethanol, which formed a crystalline oxalate. The monoester was smoothly phosphorylated with dibenzyl phosphorochloridate and the product, 2-(*N*-benzyloxycarbonyl-DL-alanyloxy)ethyl dibenzyl phosphate, was purified by silica chromatography. The oil was not characterized, but protecting groups were removed by hydrogenolysis to give 2-(DL-alanyloxy)ethyl dihydrogen phosphate (II). That this was the required compound was shown in several ways. It had the correct elemental analysis and an electrophoretic mobility consistent with the presence of an amino group and a phosphomonoester group in the same molecule. Alkali hydrolysis gave 2-hydroxyethyl dihydrogen phosphate and alanine. The ratio total phosphate: inorganic phosphate (after treatment with phosphomonoesterase):alanine was 1:0.96:1.05.

Both 2-DL-alanyloxyethanol and the dihydrogen phosphate (II) gave DL-alanine hydroxamate on treatment with hydroxylamine; the monoester (I) gave *N*-benzyloxycarbonyl-DL-alanine hydroxamate. The rates of reaction of these compounds and DL-alanine methyl ester with hydroxylamine were studied as a measure of the reactivity of their ester linkages. The results are given in Fig. 1. The dihydrogen phosphate (II) is even more reactive towards hydroxylamine than is 2-DL-alanyloxyethanol. Evidently then an adjacent phosphate group is able to increase the reactivity of an amino acid ester linkage, as does one adjacent hydroxyl group. This is in agreement with the properties of the glycerol teichoic acids; in the ribitol teichoic

Fig. 1. Reaction of esters with 0.1M-hydroxylamine at pH 7.4 and 35°. The original concentration of ester was 2 μ moles/ml.; portions (1 ml.) were withdrawn and the hydroxamate content was determined (cf. Kelemen & Baddiley, 1961). ●, 2-(N-Benzyloxycarbonyl-DL-alanyloxy)ethanol; ▼, DL-alanine methyl ester; ○, 2-DL-alanyloxyethanol; ■, 2-DL-alanyloxyethyl dihydrogen phosphate.

acids both hydroxyl and phosphate groups may enhance the lability of the ester linkages, though whether these effects are cumulative is not known.

The reaction of the dihydrogen phosphate (II) with aqueous ammonia was straightforward, giving alanine, alanine amide and 2-hydroxyethyl phosphate. The cyclic ethylene 1,2-phosphate was not detected. The slow reaction of the monoester (I) with hydroxylamine is not unexpected. Simple esters are less reactive than amino acid esters generally (Zachau & Karau, 1960).

SUMMARY

1. 2-(N-Benzyloxycarbonyl-DL-alanyloxy)ethanol, 2-DL-alanyloxyethanol and 2-DL-alanyloxyethyl dihydrogen phosphate have been synthesized.

2. The rates of reaction of the above compounds with hydroxylamine have been compared with that of DL-alanine methyl ester. Whereas the benzyloxycarbonyl derivative was less reactive, both the glycol monoester and the phosphate were

considerably more reactive than the simple ester, the phosphate more so than the glycol monoester.

3. No intermediates were detected during the reaction between the phosphate and aqueous ammonia.

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