the methyl ester of ε-carbobenzoxy-lysine yields on heating mainly ε,ε'-dicarbobenzoxy-lysine anhydride,[3] the corresponding N-carboxyl anhydride yields a polymer without any diketopiperazine. This difference may be connected with the fact that polycondensation of α-amino acid esters proceeds slowly, while the polymerization of the corresponding N-carboxyl anhydrides is rapid. Thus the methyl ester of ε-carbobenzoxy-lysine condenses to ε,ε'-di-carbobenzoxy-lysine anhydride at 105° over a period of several days, while the polymerization of ε-carbobenzoxy-α-carboxyl-lysine anhydride is accomplished under the same conditions within an hour.

Summary

ε-Carbobenzoxy-α-carboxyl-lysine anhydride (I) when heated to 102–105° evolves carbon dioxide and yields a polymeric preparation to which the structure of poly-carbobenzoxy-lysine (II) is ascribed.

The products of polymerization of (I) obtained under various conditions were studied.

On polymerization of (I) under special precautions, a poly-carbobenzoxy-lysine was obtained with an average chain length of 32-carbobenzoxy-lysine units.

Poly-carbobenzoxy-lysine (II) (*n* average = 32) yields on reduction with phosphonium iodide poly-lysine hydriodide (III) (*n* average = 32).

Poly-lysine hydriodide (*n* average = 32) contains practically no free lysine or lysine anhydride. On hydrolysis it yields lysine quantitatively.

The following derivatives were prepared from (III) (*n* average = 32): picrate, picrolonate, hydrochloride, benzoyl and 2,4-dinitrophenyl-poly-lysine.

The suggested formula for poly-lysine (*n* average = 32) is supported by the analytical data, and by the fact that 2,4-dinitrophenyl-poly-lysine (*n* average = 32) yields the expected amounts of α,ε-di-(2,4-dinitrophenyl)-lysine and ε-2,4-dinitrophenyl-lysine on hydrolysis.

Independent support for the presence of peptide bonds in (III) is given by its cleavage with crystalline trypsin.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Phosphorylation of Proteins with Phosphoric Acid Containing Excess Phosphorus Pentoxide

BY ROBERT E. FERREL, HAROLD S. OLCOTT AND HEINZ FRAENKEL-CONRAT

When proteins are treated with cold concentrated sulfuric acid, the principal reaction is a transformation of the aliphatic hydroxyl groups to half-esters of sulfuric acid.[2,3] The present study was undertaken in order to determine whether analogous reactions occur when proteins are treated with phosphoric acid containing excess phosphorus pentoxide. Levene and Schormüller[4] had used such a reaction mixture for the preparation of *o*-phosphoric acid esters of serine, hydroxyproline and serine anhydride in small yield, and Plimmer,[5] using the same medium at elevated temperatures, had duplicated their findings and also reported the preparation of *o*-phosphoric acid esters of tyrosine, threonine and isoserine.

In general, the reaction was carried out by permitting a mixture of the material to be treated and the phosphoric acid–phosphorus pentoxide reagent to stand for three days at room temperature in a dry atmosphere (desiccator). The product was isolated by pouring the reaction mixture over cracked ice, neutralizing, and dialyzing, first against ion-free water, then against 10% sodium chloride, and finally against distilled water until free of inorganic phosphates. Recoveries ranged from 70 to 100% based upon nitrogen analyses. The extent of reaction was estimated from the phosphorus-to-nitrogen ratio of the product.

Of the many polar groups in proteins available for reaction, only the aliphatic hydroxyl groups of serine, threonine and hydroxyproline, and to some extent the aromatic hydroxyl group of tyrosine were found to bind phosphorus in a stable manner.[6]

The basic groups and the peptide bonds, however, appear to be responsible for an additional amount of phosphate, retained during dialysis against water but liberated by high salt concentration. Part of the peptide bonds were labile to

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Reitz, Ferrel, Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 1024 (1946).

(3) The product obtained from wheat gluten was gel-forming and appeared to have possible industrial significance (Reitz, Ferrel and Olcott, *Ind. Eng. Chem.*, **36**, 1149 (1944)).

(4) Levene and Schormüller, *J. Biol. Chem.*, **105**, 547 (1934); **106**, 595 (1934).

(5) Plimmer, *Biochem. J.*, **35**, 461 (1941).

(6) This specificity contrasts with the non-specific action of other phosphorylating agents which are known to react with amines, guanidyl compounds, etc., as well as with alcohols and phenols. Mayer and Heidelberger[7] phosphorylated horse-serum albumin in alkaline solution with phosphorus oxychloride. The derivatives contained 2–3% phosphorus, approximately half of which was accounted for by reaction with the amino groups. The reaction of egg albumin under similar conditions was described by Heidelberger, *et al.*[8]

(7) Mayer and Heidelberger, THIS JOURNAL, **68**, 18 (1946).

(8) Heidelberger, Davis and Treffers, *ibid.*, **63**, 498 (1941).

hydrolysis when the lightly bound phosphate was present.

A considerable part of the ester-bound phosphorus was present as metaphosphate.

Reactivity of the Aliphatic Hydroxyl Groups.—That the aliphatic hydroxyl groups of proteins and model polypeptides bind phosphorus is shown in Table I, in which the amount of phosphorus introduced is compared with the β-hydroxyamino acid content. There is, in general, good correlation between the two values, although materials treated ranged in β-hydroxyamino acid content from sericin, which contains 37 equivalents per 10^4 grams (38% serine), to synthetic polypeptides containing none. Further evidence for the reactivity of the hydroxyl groups was the considerable amount of phosphorus bound by polyvinyl alcohol (18%).

The large amounts of phosphorus introduced into gelatin and isinglass were evidence that the hydroxyl group of hydroxyproline was as available for phosphorylation as was that of serine and threonine.

It appears significant in this connection that those proteins that bound amounts of phosphorus in excess of the β-hydroxyamino acids present were generally the same proteins that bound excess sulfate sulfur when treated with concentrated sulfuric acid (Table I).[2] This result may be attributed to the presence of unknown amounts of hydroxyproline, other hydroxyamino acids, or carbohydrate in the proteins. Of these, only the amount of carbohydrate can be determined with any degree of accuracy. The role of carbohydrate is illustrated by ovomucoid (26% carbohydrate), which after phosphorylation was found to contain phosphorus much in excess of the hydroxyl groups present as β-hydroxyamino acids (16.7 equivalents P/10^4 g. as compared to 7.6 equivalents β-hydroxyamino acids).

Other Polar Groups.—The phenolic hydroxyl group appears to react to a limited extent only. Model experiments with tyrosine and *p*-cresol showed that these phenols were phosphorylated to the extent of 38 and 60%, respectively, (as determined colorimetrically with the Folin phenol reagent), after treatment with the phosphoric acid–phosphorus pentoxide reagent. The original chromogenic value was restored by acid hydrolysis. Conversely, a tyrosine–formaldehyde polymer[9] containing free phenolic groups bound only trace amounts of phosphorus. The extent to which the phenolic groups of proteins participate in the reaction was difficult to evaluate. Both insulin and silk fibroin bound phosphorus in excess of their known aliphatic hydroxyl groups. These same proteins had previously been found to bind similarly high amounts of sulfate upon treatment with concentrated sulfuric acid (Table I).[2] In that case, however, model experiments with tyrosine suggested that almost no phenol sulfates were formed with the usual sulfation procedure but only sulfonates, which did not yield sulfate upon hydrolysis. That the extent of phosphorylation of the phenolic groups was small was suggested by colorimetric measurements (Folin) on intact phosphorylated insulin and untreated insulin, which were found to correspond to 8.5 and 9.0% of tyrosine, respectively. The corresponding value for the water-soluble phosphorylated silk fibroin was 6.0% tyrosine, but the fibroin derivative could not

TABLE I

COMPARISON OF STABLY-INTRODUCED PHOSPHORUS WITH THE ALIPHATIC HYDROXYAMINO ACID CONTENTS OF PROTEINS AND MODEL SUBSTANCES

	Equivalents per 10^4 g. original material		
	Phosphorus introduced[a]	Sulfate sulfur introduced[b]	Hydroxyamino acids[c]
Sericin	35.8	37.7	37.7
γ-Globulin	14.2	17.3	16.8
Gelatin	15.6	15.2	14.2[d]
Silk fibroin	16.5	16.9	12.8
Isinglass	13.7	13.7	10.2[d]
Bovine serum albumin	9.6[e]	..	10.0
Edestin	9.6[e]	..	9.3
Insulin	8.2	7.9	6.1
Globin	6.0	..	5.2
Gramicidin	6.1	5.9	4.8
Gliadin	7.4	7.9	4.6
Polyglutamic acid	0.2	1.6	..
Polyglutamine	0.3	0.7	..
Nylon	1.2[e]	0.5	..
Tyrosine–formaldehyde polymer	0.4	..	..
Polyglycine	0.8	0.5	..
Crystalline egg albumin	15.5[f]	13.6	10.0
Ovomucoid	16.7[f]	17.5	7.6
Gluten (soluble fraction)[g]	15.8[f]	7.5 (wt. av.)	5.6
Gluten (insoluble fraction)[g]	8.3[f]	..	..

[a] Calculated from the phosphorus-to-nitrogen ratio of the phosphorus derivative and the nitrogen content (dry basis) of the original material. Equivalents phosphorus introduced per 10^4 g. of original material = (% N (orig. material) P/N (derivative) × 100)/14. [b] For comparison, the results of previously reported sulfation experiments with cold concentrated sulfuric acid[2] have been included. [c] These data have been reported previously.[2] The total β-hydroxyamino acid content may be expected to be low by 10% because of the known sensitivity of serine to acid hydrolysis. Also, the method used for their determination yielded recoveries of ammonia corresponding to only about 90% of added serine or threonine. [d] The values 9.5 and 3.4 have been added for the hydroxyproline content of gelatin and isinglass, respectively. [e] In these samples significant amounts (over 10%) of the products were insoluble. Only trace amounts of phosphorus were bound in the insoluble fraction and values given here are for the soluble portion. [f] These derivatives are listed out of the normal order of decreasing hydroxyamino acid content, since they are the proteins known to contain significant amounts of carbohydrate, 26%, 10%, and 3%, respectively, for ovomucoid, gluten, and egg albumin. The role of carbohydrates in the reaction is discussed in the text. [g] Yield, approximately 50% by weight.

(9) Olcott, in preparation for press.

be compared with the original protein because of the insolubility of the latter.[10]

That the sulfhydryl groups did not react was suggested by the observation that the chromogenic activity (Folin uric acid reagent) of cysteine was not affected when this amino acid was exposed to the phosphorylating mixture. Confirming evidence for the comparative non-reactivity of the sulfhydryl groups was obtained with phosphorylated egg albumin, which, when prepared with special precautions[11] gave a strongly positive sulfhydryl test in the presence of guanidine hydrochloride. Titration indicated that slightly over half of the sulfhydryl groups were still present.

The non-participation of the guanidyl group was indicated with edestin, which contains 9.6 equivalents of guanidyl per 10^4 g. of protein. This protein bound phosphorus only to the extent of its hydroxyl groups (Table I). Further evidence was obtained with methylguanidine sulfate. No phosphorus appeared to be bound by this compound. An attempt was made to use salmine sulfate (approximately 66% arginine) as a further model. Precipitation techniques had to be used for the isolation of these reaction products. Although considerable amounts of phosphorus in excess of its hydroxyl groups were found in reacted salmine, it was possible to show that this excess was due to salt-linked metaphosphoric acid rather than to primary ester linkages (see Experimental).

That the amino group did not participate in the reaction was shown with the tyrosine–formaldehyde polymer (5% free amino nitrogen)[9] which bound almost no phosphate stably (see above). Further evidence was the fact that proteins rich in amino groups bound phosphorus only to the extent of their aliphatic hydroxyl groups, and showed unchanged or slightly increased amino nitrogen values. (Bovine serum albumin gave amino nitrogen values of 7.2 and 8.7% of the total nitrogen, respectively, before and after treatment.)

That the carboxyl group and peptide bond did not contribute to the stable fixation of phosphorus was shown with polyglutamic acid, nylon, polyglutamine and polyglycine. The labile fixation of phosphate by the peptide linkage will be discussed below.

The non-reactivity of the imidazole group was suggested by the fact that globin (approximately 9% histidine) bound phosphorus only to the extent of its hydroxyl groups (Table I).

Evidence that the indole group did not bind phosphorus was obtained with gramicidin which, although it contains almost 40% tryptophan, bound amounts of phosphorus only slightly in excess of its content of hydroxyl groups.

Nature of the Phosphate Bound.—Levene and Schormüller[4] and Plimmer[5] had found that the hydroxyamino acid phosphates prepared by reaction with phosphoric acid–phosphorus pentoxide reagent were acid esters of *o*-phosphoric acid. However, the phosphate bound in proteins and model systems appears to be a mixture of the meta and ortho and possibly other forms. Possibly the low yields obtained by these investigators were due to the presence of similar mixtures in the amino acid reaction products.

The first indication that the phosphate was not entirely in the ortho form was obtained from titration data (Table II). While synthetic monoesters of phosphoric acid and a phosphoprotein obtained from egg yolk[12] required approximately 2 equivalents of alkali for each equivalent of phosphorus present in order to bring a solution from *p*H 2 to *p*H 8, the phosphorylated proteins required only 0.7 to 1 equivalent of alkali under the same conditions.

TABLE II

EQUIVALENTS OF ALKALI REQUIRED TO TITRATE PHOSPHORYLATED PROTEINS AND MODEL SUBSTANCES FROM *p*H 2.0 TO *p*H 8.0[a]

Sample	Equivalents of alkali per equivalent of phosphorus
Phosphorylated polyvinyl alcohol	0.8
Phosphorylated gelatin	1.0[b]
Phosphorylated sericin	0.7[b]
Phosphorylated bovine serum albumin	0.8[b]
Phosvitin[d]	1.9
Sodium pyrophosphate[e]	2.1
Mono ethyl orthophosphoric acid[e]	1.9
Dimethyl orthophosphoric acid[e]	1.0

[a] Titrations were performed in 0.1 *M* potassium chloride. [b] Corrected for the titration of the untreated material over the same range. [c] Commercial preparations. [d] See footnote 12.

These findings were supported by acid group analyses by a dye method.[13] Orthophosphoric monoesters of proteins behave as dibasic acids in fixing 2 dye molecules, as was shown with the naturally occurring phosphoprotein.[12] In the artificial phosphoproteins, however, the acid group increase due to phosphorylation corresponded to only about 70% of the phosphorus, if the latter is

(10) Dispersion of both the phosphorylated and intact silk fibroin (5 mg.) in saturated lithium iodide (0.2 ml.) made a comparison possible by colorimetry immediately after dilution with water; but the accuracy of these analyses was small, since lithium iodide markedly augmented the chromogenic value of the standard tyrosine solution. The results obtained with the known samples were corrected by this apparent excess tyrosine. The tyrosine contents found by this technique were 11.5 and 12.7%, respectively, for phosphorylated and unreacted silk fibroin.

(11) In the first preparation of phosphorylated egg albumin no sulfhydryl groups could be detected. It was suspected that this might be due to the oxidizability of sulfhydryl groups of the denatured protein during prolonged dialysis in neutral solution. In an attempt to eliminate this factor, the reaction was repeated in an atmosphere of carbon dioxide and most of the acid was removed by dialysis without neutralization. The completely insoluble product was washed free of inorganic phosphorus, frozen, and dried from the frozen state *in vacuo* over sodium hydroxide flakes. Ten mg. of phosphorylated protein required 0.3 to 0.4 micromole of *p*-chloromercuribenzoate as compared with 0.7 to 0.8 for unreacted egg albumin for the abolishment of the nitroprusside test.

(12) Phosvitin, a protein containing 10.3% phosphorus: Mecham and Olcott, *Fed. Proc.*, **7**, 173 (1948).

(13) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

assumed to be present as the dibasic monoester (Table III). Therefore either most of the phosphorus introduced into proteins by this method must be present as the diester, or, more likely, a smaller fraction in an uncharged (presumably the meta ester) form.

TABLE III

APPARENT ORTHOPHOSPHATE CONTENT OF SOME PHOSPHATE DERIVATIVES BY ACID GROUP DETERMINATIONS

Samples	Equivalents per 10^4 g. original material: Acid groups: Derivative	Acid groups: Untreated	Acid groups: Increase	Phosphorus: As ortho	Phosphorus: Total
Phosphorylated bovine-serum albumin	28.8	14.6	14.2	7.1	9.1
Phosphorylated sericin	59.8	15.5	44.3	22.2	32.2
Phosphorylated polyvinyl alcohol	107.0	..	107.0	53.5	127.0
Phosvitin[a]	89.0	5.4	83.6	41.8	39.0

[a] Since phosvitin (footnote 12) is a naturally occurring phosphoprotein, the value of 5.4 acid groups in the untreated material was obtained by a determination on a 90% enzyme-dephosphorylated sample and correcting for the phosphorus still present. The other values for phosvitin are calculated on the basis of this dephosphorylated material.

To support this conclusion by elementary analytical means, polyvinyl alcohol, chosen as a simple model of a polyhydroxy compound, was phosphorylated. Complete analyses were obtained on the product, isolated both as the sodium salt and as the free acid after electrodialysis. The results corresponded to the following empirical formulas (per vinyl unit): $C_2H_{3.40}P_{0.55}Na_{0.379}O_{2.27}$, $C_2H_{3.63}P_{0.485}O_{2.04}$.

The phosphorus content indicated that about half of the vinyl alcohol units had reacted. Since titration data have shown that at *p*H 7.5 (sample neutralized to this *p*H) the ortho fraction is 90% neutralized, *i. e.*, $C_2H_{3.2}PO_4Na_{1.8}$, then the first analysis indicates that about one-fourth of the units contain orthophosphate (or the equivalent of pyrophosphate), one-third contain metaphosphate, and the rest are unreacted. The analysis of the electrodialyzed preparation indicates that about 8% of the hydroxyl groups are orthophosphate esters and about 40% are metaphosphate esters.

Salt-Labile Phosphorus.—The usual method of purifying protein derivatives by extensive dialysis was inadequate for phosphorylated proteins, in that it yielded materials having somewhat variable phosphorus contents, usually much higher than was to be expected from the β-hydroxyamino acid content of the material. In some cases phosphorus was found in model substances having no aliphatic hydroxyl groups (nylon, polyglutamine, polyglycine and tyrosine-formaldehyde polymers). This phosphorus, however, was shown to be only loosely held, since it was readily removed by dialyzing the material for five days against 10% sodium chloride (Table IV).

The results obtained with some model polypeptides (Table IV) suggested that the labile fixation of extra phosphorus might be due to the formation of salt linkages with the basic groups and to an unknown type of linkage at the peptide bond.

TABLE IV

EFFECT OF HIGH SALT CONCENTRATIONS UPON LABILE PHOSPHORUS BOUND BY SOME PROTEINS AND MODEL SYSTEMS[a]

	Equivalents phosphorus/10^4 g. original material: Before salt treatment[b]	After salt treatment[c]
Edestin	68	9.6
Sericin	41	35.8
Bovine-serum albumin	36	9.6
Polyglutamine	58	0.3
Nylon	56	1.2
Polyglycine	20	0.8
Tyrosine–formaldehyde polymer	13	0.4

[a] Dialysis for five days against 10% sodium chloride. [b] These values were not constant but varied markedly with various preparations. [c] Longer dialysis did not change these values significantly.

The participation of the peptide bonds was also suggested by Van Slyke amino nitrogen analyses on samples of phosphorylated bovine serum albumin. A preparation *not* exposed to salt dialysis (containing 36 phosphorus equivalents per 10^4 g.) seemed to contain more amino nitrogen (13.5% of the total nitrogen) than either the salt-dialyzed (8.8%) or the untreated protein (7.2%), even though no loss of nitrogen occurred during either type of dialysis. This finding could be interpreted as indicating that some peptide–phosphate bonds are labile to hydrolysis under the condition of the Van Slyke amino nitrogen analysis. Removal of the phosphate by salt dialysis restores the stability. The situation appears to be analogous to the lability toward dilute acid hydrolysis of proteins that have been exposed to concentrated sulfuric acid for several days.[2] Removal of the labily-bound sulfate by techniques that do not involve exposure to dilute acid avoids the hydrolysis. A plausible interpretation might be that phosphate or sulfate introduced on the peptide bond activates it in some manner so that under certain conditions the peptide bond is split concomitantly with the liberation of the inorganic acid.[14]

Stability of the Phosphate Bond.—A neutral solution of phosphorylated sericin was stable for four months at 4°. The phosphate bond was also stable at *p*H 2.2 and *p*H 11.5 for twenty-four hours at room temperature (23–25°). Exposure of the derivatives to 0.1 *N* hydrochloric acid and 0.1 *N* sodium hydroxide for twenty-four hours resulted

(14) Of interest in this respect is nylon, approximately 20% of which became water-soluble during the phosphorylation. This fraction contained large amounts of labily-bound phosphorus (Table IV). Subsequent salt dialysis not only liberated the phosphorus but split enough of the peptide linkages so that a major portion of the nitrogen was lost through the dialysis membrane.

in the liberation of 5–10% and 20–40%, respectively, of their phosphorus as orthophosphate, while incubation at 40° for twenty-four hours in 1% (0.25 *N*) sodium hydroxide caused the liberation of 60–80% of their total phosphorus as inorganic orthophosphate. Plimmer and Bayliss[15] and Rimington and Kay[16] reported that naturally occurring phosphoproteins were completely dephosphorylated in twenty-four hours at 37° by 0.25 *N* sodium hydroxide. Conversely, Plimmer[5] reported that the hydroxyamino acid phosphate monoesters were completely stable to both *N* hydrochloric acid and *N* sodium hydroxide at 37°.

Gel-Forming Property.—The neutral product of the reaction of wheat gluten with concentrated sulfuric acid possessed the property of absorbing large amounts of cold water rapidly to form gels.[2,3] The insoluble product obtained from wheat gluten and the phosphoric acid–phosphorus pentoxide reagent was also gel-forming but the gels were weaker than those obtainable with sulfuric acid. The hydration capacity[3] (ratio of grams water absorbed per gram gluten product) of the phosphorylated material was about 100 compared to 200–300 for gluten sulfates. Most of the products obtained from other proteins were soluble at neutrality. The insoluble fractions, except for those obtained from wheat gluten, did not form gels.

Specificity.—The observation that the phosphorylation procedure described here is even more specific for the hydroxyl groups of proteins than is sulfation with sulfuric acid suggested that the reagent might be useful for studying the role of the hydroxyl group in biologically-active proteins.[17] However, the reaction conditions are more drastic, with the result that more hydrolysis and denaturation occur. In contrast to sulfated insulin, which retains the biological potency of the untreated protein,[18] a phosphorylated insulin sample had decreased activity.[19]

Experimental

Materials.—Wheat gluten, gelatin, nylon molding powder and crystalline bovine serum albumin were commercial products. Crystalline egg albumin was prepared by the method of Kekwick and Cannan.[20] Sericin was prepared by the method of Rutherford and Harris.[21] The silk fibroin preparation was that portion of the raw silk remaining after four successive treatments with hot water. The sample of gliadin was obtained from D. K. Mecham of this Laboratory, who prepared it by fractional precipitation with alkali from dilute acid solution. Polyglutamic acid was obtained from a culture of a particular strain of *Bacillus brevis* by the method of Bovarnick.[22] The polyamide was synthesized from the peptide and has been characterized previously.[23] Protamine (salmine) sulfate and insulin were kindly furnished by the Eli Lilly Company, isinglass by the Connaught Laboratories, gramicidin by the Wallerstein Company, and edestin by D. M. Greenberg of the University of California. The tyrosine–formaldehyde polymer used was obtained by heating tyrosine with formaldehyde in acid solution.[9] The product contained approximately 5% amino nitrogen, 6.7% total nitrogen.

Phosphorylation Procedure.—Preliminary experiments showed that the following method introduced maximal amounts of phosphorus into proteins: The reagent was prepared by quickly weighing 75 g. of phosphorus pentoxide into a beaker containing 100 g. of 85% orthophosphoric acid and heating the mixture with stirring to dissolve. Ten grams of the cooled reagent was weighed into a small beaker[24] and 100 mg. of finely ground protein was dusted in with stirring to obtain a smooth dispersion. The beaker was then placed in a desiccator over phosphorus pentoxide to react for three days at room temperature.[25] The reaction mixture was stirred several times during the first twenty-four hours to disperse any lumps formed. After seventy-two hours, the viscous reaction mixture was diluted by adding finely crushed ice with vigorous stirring. The diluted mixture was then poured over more cracked ice and neutralized (*p*H 7.5–8.0) with 10 *N* sodium hydroxide, with stirring to prevent local overheating. More ice was added as needed to maintain the temperature at 5–10°. The neutralized mixture was transferred to dialysis tubing and dialyzed against running demineralized water[26] overnight and then against successive changes of distilled water until the dialysate had the same conductivity as distilled water. Some preparations were analyzed at this stage. Others were next concentrated to small volume by placing the dialyzing bags in a stream of warm air, and then dialyzing for five days against 10% sodium chloride solutions to remove all labile phosphorus. The solutions were dialyzed free of salts with distilled water, centrifuged to remove any insoluble material, frozen, and dried *in vacuo* from the frozen state. Soluble portions were either lyophilized or stored in the refrigerator after addition of a few drops of toluene to prevent bacterial action. The extent of reaction was estimated from phosphorus-to-nitrogen ratios.

Analytical Methods.—Nitrogen was determined by the Kjeldahl procedure. Total phosphorus was determined by the Allen[27] method. Inorganic orthophosphate was determined by the method of Lowry and Lopez.[28] Amino nitrogen was determined by the ninhydrin method of Harding and Maclean,[29] and by the Van Slyke manometric procedure (fifteen minutes).[30]

(15) Plimmer and Bayliss, *J. Physiol.*, **33**, 439 (1905–1906).

(16) Rimington and Kay, *Biochem. J.*, **20**, 777 (1926).

(17) Olcott and Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947).

(18) Glendenning, Greenberg and Fraenkel-Conrat, *J. Biol. Chem.*, **167**, 125 (1947).

(19) Fraenkel-Conrat and Fraenkel-Conrat, unpublished.

(20) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

(21) Rutherford and Harris, *J. Research Nat. Bur. Standards*, **24**, 415 (1940).

(22) Bovarnick, *J. Biol. Chem.*, **145**, 451 (1942).

(23) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 314 (1945).

(24) It was found advisable to use freshly prepared acid mixtures each time to prevent solidification during reaction. The composition of such mixtures has recently been elucidated, Bell, cited by Audrieth and Hill, *J. Chem. Ed.*, **25**, 80 (1948). A commercial phosphoric acid preparation containing 83–84% total phosphorus pentoxide is available under the name "phospholeum." The products obtained with this reagent were mostly insoluble (about 70%). The insoluble fractions contained very little phosphorus. The soluble fractions contained amounts of phosphorus similar to those obtained with the reagent prepared as described above (78% phosphorus pentoxide).

(25) Lower percentages of phosphorus pentoxide caused less phosphorus to be bound. Elevated temperatures could not be used to shorten the reaction time because of extensive protein degradation and losses on dialysis. The use of even traces of organic solvents as extenders and aids to dispersion of the protein led to lower phosphorus contents.

(26) Tap water could not be used, since insoluble calcium and magnesium phosphates were formed and most of the protein was rendered insoluble, complicating further purification and analysis.

(27) Allen, *Biochem. J.*, **34**, 858 (1940).

(28) Lowry and Lopez, *J. Biol. Chem.*, **162**, 421 (1946).

(29) Harding and Maclean, *ibid.*, **24**, 503 (1916).

(30) Van Slyke, *ibid.*, **83**, 425 (1929).

Tyrosine was determined colorimetrically by Herriott's modification[31] of the Folin method. Total acid groups were determined by a dye technique.[13] Cysteine was determined with the Folin uric acid reagent,[32] protein-SH groups were determined with *p*-chloromercuribenzoate[33] using nitroprusside as an external indicator.

Treatment of Low-Molecular-Weight Materials.—Several amino acids and other materials with molecular sizes too small to permit separation by dialysis were used as model systems. In general, they were handled in one of two ways: by colorimetric analyses performed on the complete, diluted reaction mixture or by measurements on the material isolated by precipitation techniques. Specific methods are cited below.

Phenols.—The reactivity of the aromatic hydroxyl group of tyrosine and *p*-cresol was ascertained as follows: 100 mg. each of tyrosine and *p*-cresol were treated for three days at room temperature with 10 g. of the phosphorylating reagent. Parallel controls (100 mg. each) were treated with 85% *o*-phosphoric acid. The reaction products were diluted with cracked ice and ice water and, in the case of tyrosine, were neutralized by the addition of the necessary amount of sodium hydroxide as calculated by titration of an aliquot to neutrality with brom thymol blue as an indicator. Tyrosine crystallized from the control solution. The diluted *p*-cresol reaction mixture had oily droplets present, hence alkali was cautiously added until these droplets were in solution. An aliquot was then titrated to neutrality and the main solution adjusted with the calculated amount of alkali. Colorimetric measurements on these solutions showed that the chromogenic values for phosphorylated tyrosine and *p*-cresol were reduced by 38 and 60%, respectively, while that for the *p*-cresol control (treated with 85% phosphoric acid) was not affected. Hydrolysis in 6 *N* hydrochloric acid at 120–125° for eighteen hours regenerated the full chromogenic activity.

Cysteine.—To ascertain whether the sulfhydryl group was phosphorylated, the amino acid cysteine was treated as follows: 100 mg. of cysteine hydrochloride was introduced into 10 g. of the phosphoric acid–phosphorus pentoxide reagent and 100 mg. was introduced into 10 g. of 85% orthophosphoric acid and run parallel as a control. The samples were allowed to react for three days at room temperature in a desiccator and then diluted with cracked ice and brought to a volume of 100 ml. with water. Nitrogen and cysteine determinations on aliquots of these solutions showed that the sulfhydryl group had not reacted.

Salmine Sulfate.—500 mg. of salmine sulfate was treated for three days with the regular phosphorylating mixture. Since it could not be dialyzed, the reaction mixture was poured into cold acetone. The water-insoluble precipitate was repeatedly dissolved in *M* sodium chloride and reprecipitated as an oil by addition of distilled water until there was no free phosphate in the supernatant fluid. The final material contained considerable quantities of phosphorus in excess of its hydroxyl groups (30.4 equivalents as compared to 4.3 equivalents per 10^4 g.), not present as free orthophosphate. However, indications were obtained that the phosphorus was present in a labile form, possibly as a salt of a metaphosphoric acid. That this explanation is a plausible one was shown with the ortho- and metaphosphoric acid salts of protamine. These were prepared by quantitatively removing the sulfate from solutions of protamine sulfate with the calculated equivalent amount of barium hydroxide and neutralizing the resulting free base with ortho- or metaphosphoric acids. The resultant orthophosphate salt showed solubility characteristics different from those of the treated material and all of its phosphorus could be determined as inorganic orthophosphate. Conversely, the meta salt had the same solubility characteristics and the same phosphorus content as the phosphorylated material, and its phosphorus could not be determined as inorganic orthophosphate.

(31) Herriott, *J. Gen. Physiol.*, **19**, 283 (1935).

(32) Anson, *ibid.*, **24**, 399 (1940).

(33) Hellermann, Chinard and Dietz, *J. Biol. Chem.*, **147**, 443 (1943).

In the light of these findings it was necessary to ascertain that the metaphosphate ion was quantitatively removed from typical proteins by our technique of dialysis against salt solution. To this end the meta salt of bovine serum albumin was prepared by acidifying a solution of the protein to *p*H 3.0 with dilute metaphosphoric acid and separating the precipitate. The 3.3% phosphorus originally present was completely removed by salt dialysis.

Methylguanidine Sulfate.—A 500-mg. sample of methylguanidine sulfate was treated in the usual manner and isolated by diluting the reaction product with approximately 4 volumes of cold acetone and pouring this diluted mixture into sufficient saturated barium hydroxide solution to keep the final mixture alkaline to brom thymol blue. The precipitate was separated and washed three times by centrifugation. Carbon dioxide was bubbled through the combined supernatants to remove excess barium. The suspension was then filtered and concentrated to a small volume. The extent of reaction was measured by nitrogen, total phosphorus, and inorganic orthophosphate determinations. No bound phosphorus was present.

Serine and Threonine.—Although the isolation in low yields of the orthophosphate esters of serine and threonine has been reported,[4,5] repeated attempts to obtain such preparations in a pure state, either by the methods previously described or by variations in the techniques of phosphorylation and isolation were unsatisfactory.

Titration Method.—The following titration method was used for the estimation of the amount of orthophosphoric acid monoester present in the phosphorylated proteins: A sample of the phosphorylated protein of suitable size to contain approximately 100 mg. original protein (calculated from nitrogen content) was placed in a 100-ml. beaker and water was added to give a volume of 18 ml. To prevent salt concentration changes from materially affecting the results, 2 ml. of *M* potassium chloride was added to make the solution 0.1 *M* with respect to salt. It was then adjusted with *N* hydrochloric acid to *p*H 2.0 as measured with a line-operated continuous-indicating *p*H meter. 0.1 *N* sodium hydroxide was then added in 0.20-ml. portions from a 5-ml. buret (graduated in 0.02 ml.) and the *p*H recorded after each addition. A titration blank was made in the same manner on an equal sample (based on nitrogen) of the untreated protein. Titration curves were prepared by plotting the equivalents of sodium hydroxide required per gram of original material against *p*H. The amount of alkali required to titrate from *p*H 2.0 to *p*H 8.0, as determined from the curve and corrected for the titration blank value, was used to determine the molar ratio of sodium consumed to phosphorus present. This value can be used then as an approximate measure of the amount of phosphorus present in the sample in the form of *o*-phosphoric acid monoester (for orthophosphate monoesters the theoretical value is 2.0). Sodium-to-phosphorus ratios for our preparations varied from 0.7 to 1.0 (Table II).

Stability.—A solution of phosphorylated sericin was stored for four months at 4° with toluene present to prevent bacterial action. It was then dialyzed against three changes of distilled water. The phosphorus-to-nitrogen was the same as that of the original derivative.

Stability at *p*H 2.2 and 11.5 was determined by adding 5 ml. of *p*H 2.2 citrate and *p*H 11.5 phosphate buffers[13] to 5-ml. samples of phosphorylated protein solutions and dialyzing the solutions against 100 ml. of the respective buffers for twenty-four hours at room temperature. The samples were then dialyzed against 10% sodium chloride for five days and finally against distilled water until the conductivity of the dialysate was equal to that of distilled water. Phosphorus-to-nitrogen ratios of the products permitted an estimate of the stability of the phosphate bonds.

Inorganic orthophosphate determinations were used to measure the extent of liberation of phosphorus in samples exposed to 0.1 *N* or stronger acid and alkali, since these conditions caused marked protein degradation leading to low nitrogen recoveries upon dialysis. Solutions of the

materials to be treated were introduced into 50-ml. Erlenmeyer flasks, and an equal volume of 0.2 *N* hydrochloric acid, 0.2 *N* sodium hydroxide, or 2% sodium hydroxide were added. The flasks were stoppered and held for twenty-four hours at room temperature in the case of the 0.1 *N* acid and alkali and at 40° in the case of the 1% sodium hydroxide. The samples were then neutralized with an equivalent amount of alkali or acid, and inorganic orthophosphate determinations were made on aliquots of the solutions.

The data obtained included only a part of the inorganic metaphosphate that may have been liberated. Control experiments with sodium metaphosphate indicated that 0.1 *N* sodium hydroxide and hydrochloric acid hydrolyzed only 11 and 22%, respectively, to orthophosphate at 23° in twenty-four hours; 28% was converted by 1% sodium hydroxide at 40° in the same length of time.

Summary

Proteins reacted with phosphoric acid containing excess phosphorus pentoxide (78% total phosphorus pentoxide) for three days at room temperature. After neutralization and dialysis, the products contained considerable amounts of phosphorus, much of which could be removed by dialysis against 10% sodium chloride solution. The remaining stably-bound phosphate was found to be present as esters of ortho- and metaphosphoric acids on the hydroxyl groups of the serine, threonine, and hydroxyproline residues. Possibly part of the phenolic hydroxyl groups, but probably no other type of protein group, participate in the stable fixation of phosphate.

The stability of the protein phosphate bonds in neutral and dilute acid and alkaline solutions has been determined.

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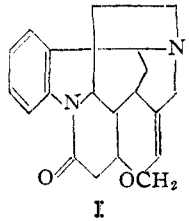
[CONTRIBUTION FROM THE CONVERSE MEMORIAL LABORATORY OF HARVARD UNIVERSITY]

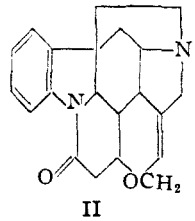
The Structure of Strychnine. Formulation of the *Neo* Bases

BY R. B. WOODWARD AND WARREN J. BREHM

Some years ago we were led to the view that strychnine was best represented by the expression[1] (I), rather than that (II) generally accepted at that time.[2] We considered *i.e.*: (i) that (I) was preferable on biogenetic grounds[3]; (ii) that (I) contains the skeletons of the main products of the drastic degradation of strychnine, *viz.*, tryptamine,[4] carbazole[5] and in particular, β-collidine,[4b,6] while the last could be formed from (II) only by rearrangement; (iii) that in any event little direct evidence was available concerning the mode of attachment of N^b to the carbazole ring.

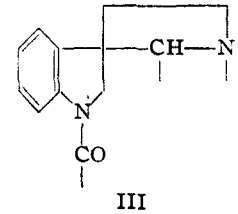


I



II

Since we have recently been able to provide evidence that the part structure (III) is present in the strychnine molecule,[7] only one major barrier



III

has remained in the way of the final acceptance of the expression (I) for strychnine. It will be clear that many of the reactions of the alkaloid will be as readily interpretable on the basis of (I) as of (II).[8] On the other hand, the acceptance of (I) has definite consequences in respect to the formulation of the *neo* series of strychnine derivatives, and the previous knowledge of the reactions of the *neo* bases has indicated strongly that these consequences did not obtain. In this communication, we describe experiments which provide conclusive proof in favor of particular expressions for relevant portions of the molecules of the *neo* bases, and show that the expressions derived are those to be expected if strychnine be formulated as (I).

The first of the *neo* bases, methoxymethyldihydro*neo*strychnidine, $C_{21}H_{24}ON(NCH_3)(OCH_3)$, was formed when strychnidine methosulfate was treated with methyl alcoholic potassium hydrox-

(1) This structure was proposed and discussed at length in the lectures by the senior author on the Chemistry of Natural Products during the summer term of 1944. It was discussed with Sir Robert Robinson in August of 1945, and since that time, investigations have been proceeding independently in the Oxford and Harvard Laboratories with the objective of final clarification of the structural situation. Professor Robinson has very generously kept us informed from time to time of the more important results of his program through private communications, and we in turn have let him know of ours.

(2) Holmes and Robinson, *J. Chem. Soc.*, 603 (1939).

(3) Woodward, *Nature*, in press.

(4) Kotake, *Proc. Imp. Acad. Tokyo*, **12**, 99 (1936); Clemo, *J. Chem. Soc.*, 1695 (1936).

(5) Perkin and Robinson, *J. Chem. Soc.*, 305 (1910); Clemo, Perkin and Robinson, *ibid.*, 1589 (1927).

(6) Clemo and Metcalfe, *ibid.*, 1519 (1937); Oechsner de Coninck, *Ann. chim.*, [5] **27**, 507 (1882); *Bull. soc. chim.*, [2] **42**, 102 (1884).

(7) Woodward, Brehm and Nelson, THIS JOURNAL, **69**, 2250 1947).

(8) A review of the enormous literature on the subject is outside the scope of this paper. An outline of the main facts is given in Henry's "Plant Alkaloids" (Blakiston's Son, 1939) and an excellent and very complete review by Professor H. L. Holmes will appear in the first volume of the forthcoming series of monographs on alkaloids, to be published by the Academic Press under the general editorship of Dr. R. H. F. Manske.