showed no depression, although this was of course not conclusive because of decomposition. The neutral equivalent was 66.3 and 67.2 (calculated, 66.1). The isomeric acids all melt at 112° and below.

Preparation of Dimethylmalonic Acid from Trimethylacetic Acid.—Pure trimethylacetic acid (20 g.), potassium permanganate (120 g.) and potassium hydroxide (10 g.) were dissolved in the minimum of water and heated seven hours on the steam-bath. The excess permanganate was destroyed with sodium bisulfite, the solution filtered, acidified, and evaporated to dryness. On acidification carbon dioxide was evolved. The solid residue was extracted with ether in a Soxhlet extractor, yielding 8.3 g. of dimethylmalonic acid. The acidified mother liquor had only a slight odor of trimethylacetic acid.

Summary

The prolonged action of hot, concentrated alkaline permanganate upon triisobutylene leaves half of the hydrocarbon unchanged. This inert component of triisobutylene has been shown to be 1,1-dineopentylethylene (II). It has been catalytically hydrogenated, converted to an epoxide and, through this and an isomeric aldehyde, to dineopentylacetic acid (V). The attacked component of triisobutylene is converted into carbon dioxide, trimethylacetic and dimethylmalonic acids.

CAMBRIDGE, MASSACHUSETTS
RECEIVED OCTOBER 17, 1940

[CONTRIBUTION FROM THE DEPARTMENTS OF MEDICINE AND BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS COLUMBIA UNIVERSITY AND THE PRESBYTERIAN HOSPITAL]

Phosphorylated Egg Albumin[1,2]

BY MICHAEL HEIDELBERGER, BARNARD DAVIS AND HENRY P. TREFFERS

Numerous chemical studies on natural phosphoproteins such as casein and vitellin[3] have furnished information of the greatest theoretical and practical importance but have afforded no basis for the comparison of these proteins with their unknown undegraded phosphorus-free analogs. Such correlation, however, would be of considerable interest, for the biological significance of phosphorylation is so far-reaching that the effect of the addition and removal of phosphoric acid groupings on the properties of proteins might well be considered. A beginning in this direction is described in the present paper.

Since crystalline egg albumin[4] is one of the most carefully studied proteins, it was decided to phosphorylate this substance. Use was made of the mild procedure adopted by Rimington[5] for the introduction of phosphorus into caseinogen, dephosphorylated (and degraded) caseinogen, and denatured serum globulin, a method based on earlier studies on lactalbumin and "blood globulin" by Neuberg and Pollak.[6]

The properties of the phosphorylated egg albumin (PEa) varied through a wide range depending upon the relative proportions of Ea and $POCl_3$ used. Products with nitrogen–phosphorus ratios of less than 30:1 were precipitable by acid below *p*H 4.5, but preparations with higher N:P ratios still showed definite differences from Ea. Physical, chemical, and immunological properties of the more representative preparations were studied and comparisons were made with Ea and certain of its derivatives.

Preparation of Phosphorylated Egg Albumin (PEa).—Crystalline egg albumin (Ea) prepared by a previously described method[7] was recrystallized three times. A typical lot contained 0.06% of phosphorus. 2.15 grams of Ea in 100 ml. of water was chilled in an ice–salt-bath at −2 to −3° and a solution of 5 g. of freshly distilled $POCl_3$ in 25 ml. of carbon tetrachloride was dropped in with mechanical stirring, during four hours. The solution, at 0–3°, was maintained at an alkalinity just pink to phenolphthalein by dropwise addition of 3 *N* sodium hydroxide. It was found necessary to add indicator from time to time, as it reacted with the $POCl_3$. When no more alkali was used up the pink color was discharged with a few drops of *N* hydrochloric acid and the mixture was centrifuged. The carbon tetrachloride layer, which had partly gelled, was washed with several small portions of water and discarded. The aqueous solution was acidified in the cold preferably with *N* hydrochloric acid to maximum precipitation after which the PEa was easily centrifuged off, leav-

(1) The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital. This study was commenced by Barnard Davis and was to have been submitted by him to the Faculty of Pure Science, Columbia University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Unfortunately the career of this engaging and promising young chemist was terminated by his tragic death.

(2) This paper was summarized in part before the New York Academy of Sciences at the Protein Symposium, February 2 and 3, 1940, and in part at the American Chemical Society Meeting, Detroit, Michigan, on September 12, 1940.

(3) References to the literature on the phosphorus linkage are cited by P. A. Levene and D. W. Hill, *J. Biol. Chem.*, **101**, 711 (1933); F. Lipmann, *Biochem. Z.*, **262**, 3 (1933); and G. Schmidt, *Z. physiol. Chem.*, **223**, 86 (1934).

(4) Subsequently referred to as Ea.

(5) C. Rimington, *Biochem. J.*, **21**, 272 (1927).

(6) C. Neuberg and H. Pollak, *Biochem. Z.*, **26**, 529 (1910).

(7) M. Heidelberger, "An Advanced Laboratory Manual of Organic Chemistry," Chemical Catalog Co., New York, N. Y., 1923.

ing a clear supernatant. When the above proportions of reactants were used this contained no unchanged Ea, as shown by tests with an anti-Ea rabbit serum. The crude PEa was resuspended in ice-cold water and dissolved with the minimum amount of *N* sodium hydroxide, solution occurring readily between *p*H 5 and 6. After six reprecipitations part of the solution was fractionated twice into portions precipitable by one-third and one-half saturation with sodium sulfate. A third fraction was obtained from the half-saturation supernatants by addition of acid. The N:P ratios of the fractions were 11.4, 10.8, and 8.9, respectively, and the amounts diminished in the same order, very little of the third fraction being obtained. The total yield of product contained 266 mg. of N, or 79% of that taken.

A portion of the unfractionated solution was again precipitated and the PEa was filtered off, washed with water, and dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets. (Lot 1). Even after one and one-half years in the dry state a sample dissolved readily when suspended in water and sodium hydroxide or ammonium hydroxide was added to neutrality.

A second lot of PEa was made similarly from 5.31 g. of five times recrystallized Ea with 14 g. of $POCl_3$ in 50 ml. of carbon tetrachloride, added during three and one-half hours. The temperature was kept below 0°. After five reprecipitations the PEa was dissolved as before, neutralized to litmus, and dialyzed in the cold under negative pressure in a cellophane tube against distilled water in the presence of toluene. Considerable free phosphate was eliminated in this way. The resulting solution contained 1.81 mg. of N and 0.336 mg. of P per ml., making the N:P ratio 5.4. Products containing so large an amount of P could not be obtained in subsequent preparations even when four times as much $POCl_3$ was used. While the reasons for this are not entirely clear, it was found that a part of the P in PEa with N:P ratios of about 9 was in very labile form, varying amounts being split off when the experimental procedure for isolating PEa was varied. For instance PEa 7A (Table I) was reprecipitated three times in the cold at *p*H 2.99, 2.88, and 3.18, respectively, redissolved, and dialyzed against 0.9% NaCl solution in the cold until the amount of P in the outside fluid reached an apparently constant minimum. The entire process required one week and the N:P ratio was then 8.7 and the *p*H 7.24. Two weeks later a portion of the solution kept at 0–5° was reprecipitated and the supernatant was found to contain phosphate equivalent to 10% of the P in the PEa used while a portion held at 37.5° in the presence of toluene during the second week lost 61% of its P.

Another portion, 7I, of the original solution of Preparation 7, was dialyzed in the cold, in the presence of toluene, against 0.9% sodium chloride, without precipitation with acid. Two weeks were required to reach a relatively constant minimum of $PO_4^{\equiv}$ in the outside fluid. The N:P ratio of PEa 7I was then 8.9 (Table I), practically equal to that of the portion, 7A, which had been purified more rapidly by several reprecipitations with acid. Accordingly precipitation with acid, if carried out in the cold, does not lower the P content.

Fraction 7B was isolated by saturation of the neutralized supernatant from 7A with sodium sulfate at 37°, solution of the precipitate in water and precipitation with acid to maximum turbidity.

TABLE I

PROPERTIES OF PHOSPHORYLATED EGG ALBUMIN

Lot	Basic ash (as Na), %	N,[a] %	P,[a] %	N:P ratio	Approx. no. of phosphoryl groups in prepn.[b]	N pptd. by 0.043–0.047 mg. antigen N from anti-PEa serum, mg.	N pptd. by 0.09 mg. antigen N from anti-Ea serum,[c] mg.
1	0.1	14.6	1.52[d]	9.6	18		
2			2.71[d]	5.4	32		
5A				28	6	0.18	0.22
B				66	3		.81
C				76	2	.03	.83
D				90	2		.85
6A	.6	14.8		12.5	14	.26	.08
7A				8.7	20		
I				8.9	19		
B				17	10		
8A				9.4	18	.23	.10

[a] Calcd. to the ash-free basis. Lot 1 contained 1.67% S and was originally precipitated with HOAc instead of HCl. [b] Using 36,000 as the molecular weight of PEa. [c] Maximum N precipitable by Ea, 0.83 mg. [d] From N:P ratio, taking N = 14.6%.

Preparation 8A (Table I), from Ea prepared and recrystallized four times according to Kekwick and Cannan,[8] required a longer period of dialysis than 7A and showed an N:P ratio of 9.4. The *p*H was 7.20. After another week in the cold precipitation with acid (at *p*H 3.05) and analysis of the supernatant showed that 13% of the P had been liberated as $PO_4^{\equiv}$, while five days at 37.5° resulted in the elimination of a total of 60%. The PEa precipitated by acid (at *p*H 4.04) in the experiment at 37.5° was redissolved in 0.9% saline with 0.1 *N* sodium hydroxide and, at *p*H 7.34, was again allowed to stand for one week at 37.5° in the presence of toluene. This time after precipitation with 0.1 *N* hydrochloric acid (at *p*H 4.22) an additional 8% of $PO_4^{\equiv}$ was found in the supernatant. The precipitate was redissolved in saline with a little alkali and dialyzed against saline, after which the N:P ratio was 26, while the ratio calculated from the amount split off was 29. Removal of the labile phosphorus failed to change the amount of antibody precipitated by PEa 8A (see Table I) from anti-Ea or anti-PEa serum.

Preparation 6, otherwise comparable to 7, was allowed to stand for seventeen days before working up. After four precipitations with acid (*p*H of supernatants, 3.38, 3.65, 3.65, and 3.74) the supernatant was practically free from protein and $PO_4^{\equiv}$. Dialysis was completed four weeks from the phosphorylation reaction, and the N:P ratio of the main product, 6A, was 12.5.

In preparation 5, an attempt was made to phosphorylate Ea to a slight degree. The reaction was carried out with 1.5 g. of Ea and 0.5 g. of $POCl_3$ in 10 ml. of carbon tetrachloride—otherwise the conditions used were similar to those of the other preparations. Maximum precipitation of the PEa was found to occur at about *p*H 4.2, but the product, 5A, dissolved readily in excess acid, contrary

(8) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936)

to the behavior of the more highly phosphorylated lots. After solution in chilled saline with 0.1 *N* sodium hydroxide to neutrality and reprecipitation three times, followed by dialysis, PEa 5A showed an N:P ratio of 28:1. The supernatant from 5A was precipitated by one-half saturation with sodium sulfate and addition of a few drops of 0.2 *N* sulfuric acid, finally yielding, in addition to water-insoluble material, a water-soluble fraction 5B, precipitable by half-saturation with sodium sulfate in neutral solution, and a portion of 5C, requiring slight acidification for precipitation. An attempt was made to crystallize Fraction 5C, with N:P ratio 76, in the hope that any unchanged Ea present might be removed in this way. Although seeded with Ea crystals under conditions favorable for the crystallization of Ea the fraction remained amorphous. The separation of Ea in the Tiselius electrophoresis cell[9] was also attempted at *p*H 6.10, but only a single boundary was observed, with a mobility (descending) of 7.7×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, a value considerably higher than that of Ea under the same conditions.

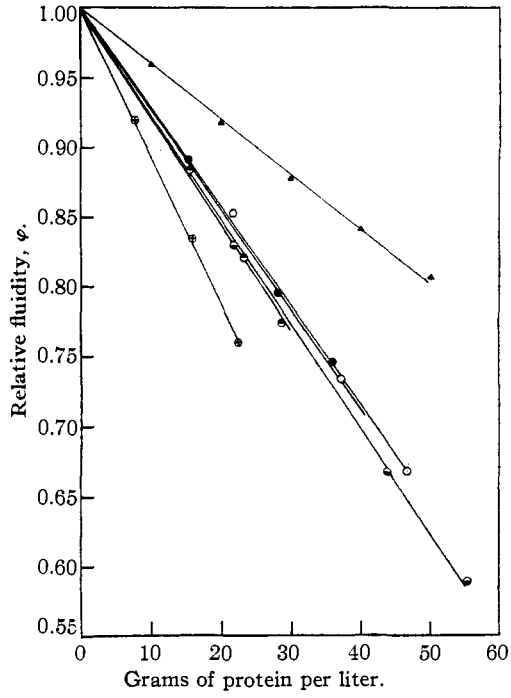


Fig. 1.—Fluidities of phosphorylated egg albumin solutions in 0.15 *M* NaCl at *p*H. 6.6: ○, prepn. 8D; ●, prepn. 6A; ◒, prepn. 7A; ◓, prepn. 8A; ⊕, prepn. 5A; ▲, comparison curve of egg albumin.[13]

Analyses of the various preparations for phosphorus were made by the Pregl–Lieb method, except in the case of some of the splitting experiments, in which phosphate was estimated according to Bodansky.[10]

Stability of PEa to Alkali.—According to Plimmer and Bayliss[11] P may be completely removed from phosphoproteins such as casein by 1% sodium hydroxide at 37° for twenty-four hours. This treatment split off only 50% of the P in Preparation 6A. Seventy per cent. of the protein remained insoluble on acidification. This material failed to react with anti-Ea serum but precipitated one-third as much N from anti-PEa serum as did a comparable amount of untreated 6A.

Reactions with Cations.—A 1% solution of preparation 8D, from which P labile at 37° had been removed, gave immediate precipitates with copper, nickel, cobalt, and cadmium salt solutions the acidity of which had been reduced sufficiently to prevent precipitation of PEa by H^+. Of these solutions only Cu^{++} precipitated Ea and Ni^{++} gave a slight turbidity.

Solubility of PEa in Acid.—In the determination of the titration curve PEa 6A was precipitated with hydrochloric acid and brought to *p*H 1.8 without marked solution taking place. However, when a saline solution of PEa 6A which had stood for several months and lost a portion of its combined P was precipitated with dilute acetic acid and then treated with *N* hydrochloric acid the turbidity diminished somewhat. On dilution with water the protein dissolved. A suspension of PEa 8A behaved similarly.

Incoagulability of PEa Solutions on Boiling.—Neutral solutions of PEa in saline remained clear on boiling. Under similar conditions Ea coagulated readily but denatured egg albumin did not.

Viscosity and Fluidity of PEa Solutions.—Portions of various lots of PEa at *p*H 6.6 (measured at room temperature) were dialyzed in the cold against 0.9% sodium chloride and readjusted to *p*H 6.6 if necessary. Viscosities of several dilutions of each solution were measured at 25.0° in an Ostwald viscometer of 2.0 ml. capacity. All solutions were weighed, but the concentrations are given in g. of protein per l. Measurements are given in Fig. 1 in terms of the fluidity, φ, or reciprocal of the relative viscosity of the protein solution to that of the solvent, 0.9% sodium chloride. This function has been shown to be linear with respect to concentration for most protein solutions.[12] The data may be expressed by the equation $\varphi_{rel.} = 1 - kC$, in which C is the protein concentration in g. per l., and k is the slope. The N:P ratio was redetermined for most of the solutions, and the protein concentration calculated from the N concentration and the theoretical N percentage for the N:P ratio found. The N:P ratio at the time of the experiment and the slope of the fluidity line as determined by the method of least squares[12] were 28 and 0.0106 for preparation 5A, 13.6 and 0.00717 for preparation 6A, 12.1 and 0.00732 for preparation 7A, 11.5 and 0.00771 for preparation 8A, and 26 and 0.00710 for preparation 8D. For comparison the viscosity data of Polson[13] on egg albumin (Ea) in 0.2 *M* sodium chloride at *p*H 6.67 have been recalculated in terms of fluidity.

Titration Curve of PEa.—A portion of lot 6A solution was dialyzed in the cold against 0.15 *M* potassium chloride solution and allowed to stand several days under slight negative pressure in the presence of 0.15 *M* potassium hydroxide to remove any carbon dioxide present. Five ml. of the solution, at *p*H 6.07 and containing 40 mg. of PEa, was titrated in a nitrogen atmosphere with 0.02

(9) A. Tiselius, *Nova Acta Regiae Soc. Sci. Upsaliensis*, IV, **7**, No. 4 (1930); *Biochem. J.*, **31**, 1464 (1937).

(10) A. Bodansky, *J. Biol. Chem.*, **99**, 197 (1932–1933).

(11) R. H. A. Plimmer and W. M. Bayliss, *J. Physiol.*, **33**, 439 (1905–1906).

(12) H. P. Treffers, THIS JOURNAL, **62**, 1405 (1940).

(13) A. Polson, *Kolloid Z.*, **88**, 58 (1939).

ml. increments of 0.1 *M* sodium hydroxide or 0.1 *M* hydrochloric acid using the differential technique with glass electrodes,[14] one of which was kept at constant *p*H with buffer solution. The lower limit (*p*H 5.0) of the titration with acid was set by the formation of a precipitate in the solution. Absence of carbon dioxide was checked after titration on a 1-ml. portion in the Van Slyke apparatus.

A solution of Ea[8] was titrated similarly, and the titration curves for the two proteins were set equal at *p*H 5.06 (Fig. 2). The curve for Ea gives the combining capacity per g. of protein, that for PEa the combining capacity of an amount (1.03 g.) equivalent to 1 g. of Ea. At *p*H 8.0 the combining capacity of PEa 6A was 29×10^{-5} eq. greater than that of Ea, while at *p*H 9.5 the difference was 37, in satisfactory agreement with the value, 36, calculated on the assumption that all of the phosphoryl groups introduced function as monobasic acids over this range. That their properties are somewhat modified by combination with the protein may be indicated by the non-parallelism of the two curves on the alkaline side of this range.

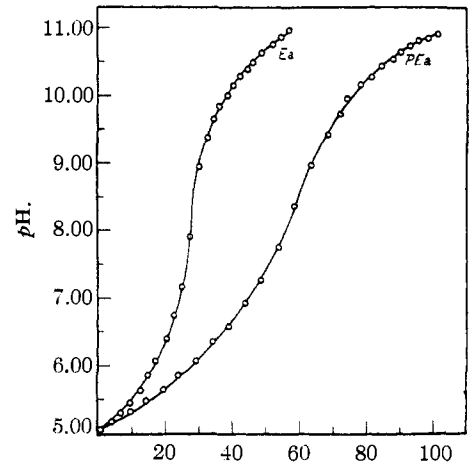
Immunological Properties of PEa.—Rabbits 9.00 and 9.01 were injected repeatedly with small doses of alum-precipitated PEa (Lots 1 and 2), in the same way as with Ea,[15] except that somewhat smaller amounts were used. As shown in Table II the PEa-anti-PEa reaction resembled the Ea-anti-Ea precipitation[15] and other homologous protein-antiprotein reactions already studied[16] except that there was no clean-cut equivalence zone in which tests on the supernatants for both components were negative.

TABLE II

ADDITION OF INCREASING AMOUNTS OF PEa, Ea, AND DnEa[a] TO ANTI-PEa RABBIT SERUM, 0°, FORTY-EIGHT HOURS

PEa N added, mg.	Nitrogen pptd., mg. Total	PEa	Anti-body	Ratio Anti-body N to PEa N in ppt.	Tests on supernatants: + PEa (test for anti-body)	+ Serum (test for anti-gen)
		Serum 9.01_1, 1.0 ml.				
0.026[b]	0.251	(0.025)	(0.226)	(9)	+	±
.053	.36				+	++
.105	.40				±	++
.210	.37					
.420	.25					
		Serum pool 9.00_1 + 9.01_2, 1.0 ml.				
.013[c]	.14	0.01	0.13	10	+	±
.026	.22				−	+
.052	.20					
Ea N added						
0.054	.01					
.268	.01					
DnEa[a] N added						
0.056	.11					
.280	.28				+	±
1.120	.40				−	+

[a] Acid-denatured Ea. [b] Lot 1. [c] Lot 2.



Equivalents KOH bound ($\times 10^5$) per gram of protein.

Fig. 2.—Alkaline titration curves of egg albumin and of phosphorylated egg albumin, prepn. 6A.

The reciprocal cross-reactions, Ea-anti-PEa and PEa-anti-Ea, were slight and involved not more than 3 to 10% of the antibody in the sera tested. Further indication of the radical change in specificity on passage from Ea to PEa was furnished by the failure of Ea to inhibit precipitation of PEa in anti-PEa serum, 100 times as much Ea as PEa necessary to reach the equivalence zone being ineffective.

As also noted in Table II the cross-reaction of acid-denatured Ea[17] with anti-PEa is so marked that all of the antibody is precipitated when large amounts of DnEa are used. The reverse reaction, that of PEa in anti-DnEa sera, is also a strong one. The DnEa-anti-PEa reaction, originally studied on account of superficial resemblances between DnEa and PEa, showed that the resemblances were quite marked although the reaction followed a course different from the homologous PEa-anti-PEa and DnEa-anti-DnEa reactions.[18] It therefore became imperative to determine to what extent the supposedly mild preparative conditions contributed to the shift in properties toward those of DnEa.

Blank Preparation without $POCl_3$.—620 mg. of Ea[8] in 40 ml. of H_2O and 15 ml. of 6% aqueous Na_2HPO_4 were chilled as in the phosphorylations. After addition of 20 ml. of carbon tetrachloride the mixture was stirred and 30 ml. each of 3 *N* sodium hydroxide and hydrochloric acid were dripped in over a period of one and one-half hours in such a way that the reaction remained just alkaline to phenolphthalein except for occasional momentary intervals. The mixture was allowed to stand overnight and the aqueous solution was acidified with 3 *N* hydrochloric acid to maximum turbidity (*p*H 4.0). Nitrogen determinations on the precipitate and supernatant showed that one-third of the Ea had become denatured. The solution of the precipitate reacted heavily with anti-DnEa serum.

Discussion

It is evident from the data presented that up to about 20 to 30 phosphoryl groups may be in-

(14) D. MacInnes, "Principles of Electrochemistry," Reinhold Publishing Co., New York, N. Y., 1939.

(15) M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **62**, 697 (1935). Details of the analytical methods used will be found in this article.

(16) For reviews, *cf.* M. Heidelberger, *Chem. Rev.*, **24**, 323 (1939); *Bact. Rev.*, **3**, 49 (1939).

(17) Subsequently referred to as DnEa.

(18) For a preliminary note see C. F. C. MacPherson and M. Heidelberger, *Proc. Soc. Exptl. Biol. Med.*, **43**, 646 (1940).

troduced readily into crystalline egg albumin and that the introduction of these polar groups is accompanied by a remarkable series of changes in the chemical, physical and immunological properties of the protein. These are a loss of heat-coagulability, a greatly increased buffering capacity in the neutral range, an increase in viscosity, acquisition of precipitability by salts of nickel, cobalt and cadmium and a radical change of immunological specificity without loss of antigenic properties. Unfortunately the preparative conditions involved a denaturation of a portion of the Ea, so that the various preparations must be regarded as mixtures of PEa and PDnEa, probably in varying proportions. It is therefore uncertain to what extent the changes noted in some of the properties are due to denaturation rather than to phosphorylation. A parallel study with the less easily denatured crystalline horse serum albumin, now in progress, should throw light on this question.

The nature of the groupings occupied by the entering phosphoryl radicals is of the greatest interest, but is not settled by the present experiments. The only amino acid in casein known to be phosphorylated is serine[3] although Rimington believed he had isolated a phosphorylated peptide containing hydroxyglutamic acid and hydroxyaminobutyric acid as well as serine.[19] The number of hydroxyamino acid residues in the Ea molecule could scarcely equal 30. Since Ea contains carbohydrate it is possible that this may be phosphorylated even under the mild conditions used, so that the carbohydrate and hydroxyl groups of the hydroxyamino acids might together account for the large number of phosphoryl groups that may be introduced. It is uncertain whether or not the —OH groups of the tyrosine radicals in Ea react, for though PEa gave less color with the Folin phenol reagent than did Ea, no marked difference could be noted in the extent of coupling of Ea and PEa with diazotized sulfanilic acid under comparable conditions with an excess of sodium carbonate. It is also uncertain whether or not the amino groups of the lysine or the guanidine residues of the arginine in Ea would be phosphorylated under the conditions used. Possibly the more labile phosphoryl groups present are attached in this way or even to carboxyl in anhydride form as suggested by Lipmann.[20] Further experiments on the elucidation of the mode of attachment of the P are planned, particularly as such studies may facilitate the identification and estimation of the hydroxyamino acids in proteins.

The data given in Fig. 1 for preparations 6A, 7A, and 8A indicate a close connection between the content of phosphoryl groups and viscosity, as the fluidities vary in the order of the N:P ratios. Also, in a given preparation (8A) removal of the labile phosphoryl groups is accompanied by a decrease in viscosity (increase in fluidity) (8D). Some other factor also intervenes, for the most lightly phosphorylated preparation, 5A, was the most viscous. Possibly the extent of denaturation is of influence, although this effect should have been minimal in Lot 5A. All of the PEa solutions were far more viscous than those of Ea.

The great increase in buffering power of PEa over Ea in the neutral range (Fig. 2) may legitimately be ascribed to the phosphoryl groups introduced, especially as the titration curves of Ea and acid-denatured Ea are almost identical.[21] The additional base combined is approximately that calculated for the number of phosphoryl groups present.

The immunological findings permit several conclusions to be drawn. The almost complete failure of Ea to precipitate anti-PEa sera and the similar lack of reactivity of PEa in anti-Ea sera indicate that phosphorylation with a measure of accompanying denaturation induces as profound a change in molecular structure as is noted in the denaturation of Ea by acid.[18] Owing to the antigenicity of PEa the change in specificity cannot be ascribed to degradation induced by the phosphorylation procedure, nor can the altered specificity be due to the blocking of immunologically reactive groupings in the Ea molecule by entering phosphoryl groups, as in such a case the reciprocal cross reactivity with the DnEa system would scarcely be so marked.

However, precipitation of DnEa by anti-PEa serum is actually a cross reaction and is not due to the presence of DnEa in the PEa. The reaction is very different from that in the homologous PEa-anti-PEa (Table II) and DnEa-anti-DnEa[18] systems in that amounts of antigen of an entirely different order of magnitude are required for precipitation of all of the antibody and in that the

(19) C. Rimington, *Biochem. J.*, **21**, 1179, 1187 (1927).

(20) F. Lipmann, *Nature*, **144**, 381 (1939); *J. Biol. Chem.*, **134**, 463 (1940).

(21) Private communication from Prof. R. Keith Cannan.

total amount of N precipitated may greatly exceed that thrown down in the homologous reaction. Similar behavior has been noted by Kleczkovsky[22] in cross reactions of chemically altered horse serum globulin in antisera to unaltered globulin but not with Ea and its corresponding derivatives and their antisera, and was ascribed to a physical alteration in the antigen preventing the formation of soluble complexes with antibody. This cannot apply in the case of DnEa since excess of this antigen results in an inhibition zone with anti-DnEa. The failure of the DnEa-anti-PEa system to show inhibition with excess DnEa is more probably due to a relatively restricted range of combining proportions and a greater tendency of any soluble compound formed to dissociate.[23]

The specificity conferred upon Ea by the introduction of phosphoryl groups is not entirely a hapten specificity in the sense of Landsteiner[24] for anti-PEa sera are not precipitated by phosphates nor is the specific precipitation of PEa inhibited by as much as 0.15 *M* phosphate. On the other hand, one of three lots of phosphorylated Felton solution (water-precipitable globulin from anti-pneumococcus horse serum) and one lot of phosphorylated horse serum albumin[25] gave precipitates with anti-PEa but casein (Merck) did not.

(22) A. Kleczkovsky, *Brit. J. Expll. Path.*, **21**, 1, 98 (1940).

(23) *Cf.* M. Heidelberger and F. E. Kendall, *J. Expll. Med.*, **59**, 519 (1934).

Summary

The phosphorylation of crystalline egg albumin and its effect on the viscosity, titration curve, and other physical, chemical and immunological properties of the product are described and discussed. The lability of a portion of the phosphorus is noted and commented upon.

(24) K. Landsteiner, "The Specificity of Serological Reactions," C. C. Thomas, Springfield, Ill., 1936.

(25) Immunochemical studies on these products are now in progress.

NEW YORK, N. Y. RECEIVED OCTOBER 26, 1940

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE, AND THE GEORGE WASHINGTON UNIVERSITY SCHOOL OF MEDICINE]

Purification of the Pressor Principle of the Posterior Lobe of the Pituitary Gland by Electrophoresis

BY GEORGE W. IRVING, JR.,[1] HELEN M. DYER AND VINCENT DU VIGNEAUD

In a recent communication a simple method was presented by which fairly large amounts of a preparation containing 10 pressor and 10 oxytocic units per mg. could be readily obtained from the posterior lobe of the pituitary gland.[2] In a continuation of these preparative studies it was decided to explore the possibility of utilizing electrophoresis for purification of these principles. Our previous investigation of their electrophoretic behavior had demonstrated that under the conditions employed both migrated toward the cathode, the pressor principle traveling at a faster rate.[3,4] This work had given rise to the hope that by subjecting the material to electrophoresis, various impurities might be separated from the active material. The present communication deals with the development of such a preparative method for the pressor principle. A crude preparation, the "ether precipitate" previously described,[2] was partially purified by fractionations with various solvents and the resulting preparation was then subjected to electrophoresis in a newly designed apparatus. By this procedure 35 mg. of a preparation containing 200 pressor units per mg. was obtained per kilo of posterior lobes.

Since evidence has been presented which indicates that the pressor principle undoubtedly contains the disulfide linkage,[5,6,7] the cystine content of a series of fractions was determined to ascertain the effect of purification upon the content of this amino acid. Tyrosine analyses were carried out for the same reason since tyrosine also has been shown to be present in fairly high concentration in highly potent pressor preparations.[8,9]

Experimental

Prevention of Inactivation in the Cathode Cell.—Under certain conditions, inactivation of the posterior lobe prin-

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