ROLE OF CATION IN THE DEPHOSPHORYLATION OF PHOSPHOPROTEINS BY ALKALI

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The alkali-labile nature of phosphoprotein phosphorus was first recognized by PLIMMER AND BAYLISS[1], who observed casein to be readily dephosphorylated by dilute alkali at room temperature. The mild conditions under which dephosphorylation occurs were found by later investigators to be characteristic of phosphoproteins[2,3]. This property has also found extensive application in recent years for following the turnover of phosphoprotein phosphorus in various tissues[4–6]. There have been, however, very few investigations dealing with the chemical aspects of this reaction and not much is known with regard to the effect of various factors such as the pH and the nature of the cation on the dephosphorylation reaction. The present investigation is concerned with a detailed study of these aspects and deals further with a study of the kinetics of the dephosphorylation of various phosphoproteins. The preparation of alkali-dephosphorylated casein through the use of a mild dephosphorylating agent and a chemical method for the preparation of casein phosphopeptone have also been described in the present communication.

MATERIALS AND METHODS

Protein substrates

Phosvitin and lipovitellin were prepared from egg yolk following respectively the procedures of MECHAM AND OLCOTT[7] and ALDERTON AND FEVOLD[8]. Ovalbumin was a twice-crystallized product prepared according to KEKWICK AND CANNAN[9]. Dephosphorylated casein was prepared by an enzymic method[10]. α- and β-casein samples prepared according to HIPP and co-workers[11] were kindly supplied by Dr. T. L. MCMEEKIN (U.S.A.). Pepsin (twice-crystallized, Worthington Biochemical Corporation, U.S.A.) and bovine plasma albumin (Armour Laboratories, U.S.A.) were commercial products while human blood fibrin was a generous gift from Haffkine Institute, Bombay.

Incubation

The incubation mixture employed in the dephosphorylation experiments was usually made up of a solution of the sodium salt of the protein in the desired alkali and was present in a final volume of 1 ml. The protein solution and the alkali were preincubated separately at 37° and the reaction was started by adding sufficient of the alkali to the protein to bring the final concentration of the alkali to the desired value. Incubations were carried out at 37° in stoppered test tubes and the reaction was stopped at the end of the desired time interval by the addition of 4 ml of a solution containing a mixture of trichloroacetic acid (5% w/v) and sulphuric acid (0.0625 *N*). The protein precipitate was brought into a homogeneous suspension and the precipitate removed by centrifugation.

Total and inorganic phosphorus determinations were carried out according to the method described by FISKE AND SUBBAROW[12]. Acid-soluble nitrogen in the trichloroacetic acid filtrates was estimated according to the method of KOCH AND MCMEEKIN[13], with suitable modifications for the estimation of microgram quantities of nitrogen.

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References p. 158.

RESULTS

Dephosphorylation of phosvitin by metal hydroxides

The dephosphorylating action of various alkalis on phosphoproteins was studied, employing the hydroxides of the alkali and alkaline earth metals. Phosvitin was used as substrate in these studies because of its high content of phosphorus. The protein was taken in a final concentration of 2 mg per ml of the incubation mixture. Owing to the very limited solubility of $Ca(OH)_2$ in water, the maximum concentration attainable with this reagent was only about 0.025 *N* and all the other alkalis were accordingly employed at the same concentration. From the results presented in Fig. 1, it can be readily seen that the hydroxides of Ca^{++}, Ba^{++}, and Sr^{++}, are extremely active as dephosphorylating agents, $Sr(OH)_2$ being the most effective in this respect. NaOH, at the concentration employed, was rather ineffective. Ammonia had no dephosphorylating activity and even at high concentration (1 *N*) could split only 5% of the protein phosphorus in 24 h.

These results show clearly that alkalis vary sharply in their dephosphorylating activity. The difference in activity may be attributed either to the nature of the cation present in them or to a difference in the pH of these reagents. To test the latter possibility, solutions of the protein in the various alkalis were made up exactly as was done for the dephosphorylation experiments and the pH of the resulting solutions were measured in a Beckman pH meter. The results presented in Table I are such as to rule out the possibility of pH being the sole factor influencing the extent of dephosphorylation and focus attention on the role of the cation in this reaction.

TABLE I

pH OF SOLUTIONS OF PHOSVITIN IN ALKALI

0.2% (0.3% in the case of ammonia) solution of phosvitin in various alkalis was made up and pH measured in a Beckman pH meter.

Alkali	*Strength of alkali (Normality)*	*pH of solution*
Strontium hydroxide	0.025	11.5
Barium hydroxide	0.025	11.6
Calcium hydroxide	0.025	11.6
Sodium hydroxide	0.025	11.7
Ammonia	1.000	10.9

These findings led naturally to a study of the influence of various metal ions on the dephosphorylation reaction. In these experiments, the protein (3 mg per ml) was preincubated with several metal ions (0.1 *M* final concentration) for a period of 15 min and the reaction was started by the addition of ammonia to 1.0 *N* final concentration. Ammonia served the dual purpose of providing the necessary alkalinity to the medium, while having, by itself, no significant dephosphorylating action. Incubations were carried out in these experiments for a period of 24 h. The results shown in Table II present several interesting features. The addition of sodium chloride had no appreciable effect on the reaction. As was to be expected, dephosphorylation was increased to a marked extent in the presence of Ba^{++}. Mg^{++}, though belonging to the same group, was not so effective. Mn^{++}, Co^{++}, and Fe^{++}, which form the

References p. 158.

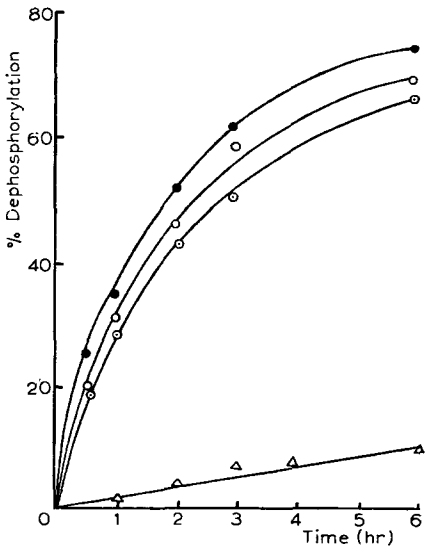

Fig. 1. Dephosphorylation of phosvitin (177.0 μg protein phosphorus) by 0.025 *N* alkali: $Sr(OH)_2$ (●—●—●), $Ba(OH)_2$ (○—○—○), $Ca(OH)_2$ (⊙—⊙—⊙) and NaOH (△—△—△).

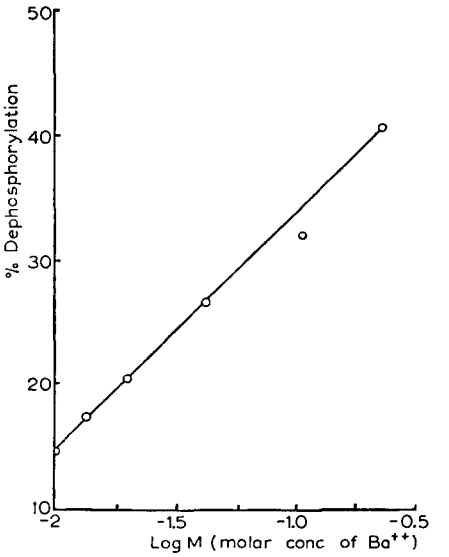

Fig. 2. Dephosphorylation of phosvitin (266 μg protein phosphorus) as a function of cation concentration.

TABLE II

EFFECT OF SOME CATIONS ON THE DEPHOSPHORYLATION OF PHOSVITIN BY AMMONIA

Phosvitin (3 mg/ml) was preincubated with indicated metal ion (0.1 *M* final concentration) for 15 min and then treated with ammonia to 1 *N* concentration. Incubation at 37° for 24 h.

Metal ion	*Inorganic phosphorus liberated (μg)*
No addition	14
Na^+	16
Mg^{++}	49
Ba^{++}	164
Mn^{++}	41
Fe^{++}	56
Co^{++}	30

neighbouring group of elements, produced significant activation. Mo^{+++}, which is known to catalyse the hydrolysis of several low-molecular-weight phosphate esters in acid medium, had, however, no effect on this reaction. Cu^{++}, Zn^{++}, Ni^{++}, and Al^{+++}, were similarly ineffective (not shown in the Table). These results show conclusively that the dephosphorylation of phosphoproteins by alkali is influenced to a large extent by the nature of the cation present in the medium.

In Fig. 2 is shown the relationship between the molar concentration of cation and its dephosphorylating action. In these experiments, the protein (3 mg per ml) was preincubated, for a period of 15 min, with increasing concentrations of barium chloride before the addition of ammonia. Incubation was carried out for a period of 4 h. The extent of dephosphorylation was observed to be proportional to the

References p. 158.

logarithm of the molar concentration of barium chloride, at the range of concentrations employed in these experiments. Activity was too low at concentrations of Ba^{++} less than 0.01 *M*.

Action of barium hydroxide on other phosphoproteins

Dephosphorylation of pepsin and ovalbumin. The kinetics of the dephosphorylation reaction, catalysed by alkali, was next investigated, employing as substrates a variety of phosphoproteins containing phosphorus linked in different ways. Barium hydroxide at a final concentration of 0.25 *N* was employed as dephosphorylating agent in these investigations. The results obtained with pepsin and ovalbumin as substrates are presented in Table III. The single phosphorus atom of pepsin appears to be readily split off, maximum dephosphorylation occurring within an hour. With ovalbumin, about 50% of its phosphorus was cleaved within 30 min, while the rest of it appeared to be resistant to dephosphorylation and required a prolonged incubation period for removal from the protein. Similar results have been obtained by PERLMANN[14] and FLAVIN[15] on the enzymic dephosphorylation of ovalbumin. These workers found that 46% of the protein phosphorus was readily removed by various phosphatases. It is quite probable that the phosphorus grouping susceptible to hydrolysis is the same in all the cases.

TABLE III

DEPHOSPHORYLATION OF PEPSIN AND OVALBUMIN BY ALKALI

Total protein phosphorus: 20 μg in the case of pepsin and 40 μg in the case of ovalbumin.

Time of incubation (hours)	*% Inorganic phosphorus released*	
	Ovalbumin	*Pepsin*
0.25	38	—
0.50	51	62.5
1.00	55	81.0
3.00	72	81.0
24.00	100	—

Dephosphorylation of phosphoproteins of egg yolk. The dephosphorylation of phosvitin and lipovitellin is indicated in Fig. 3. With the former substrate the reaction followed a linear pattern till about 75% of the phosphorus was split off. Hydrolysis of the rest of the phosphorus proceeded at a markedly slower rate. In the case of lipovitellin, maximum dephosphorylation of about 50% of its phosphorus was obtained within 1 h and there was no further increase in phosphorus release even after 24 h of incubation. Assuming that 18% of the lipoprotein constituted phospholipid[8], the maximum dephosphorylation observed in this case corresponds to nearly 95% of the vitellin phosphorus.

Dephosphorylation of casein. Dephosphorylation of α- and β-casein as well as of unfractionated casein is shown in Figs. 4–6. Dephosphorylation observed with NaOH and KOH are also shown for purpose of comparison. It may be seen that the kinetics of dephosphorylation is similar with all the proteins. About 75% of the phosphorus was readily split off by barium hydroxide in 3 h, after which the reaction slowed down considerably. Dephosphorylation by the hydroxides of sodium and potassium

References p. 158.

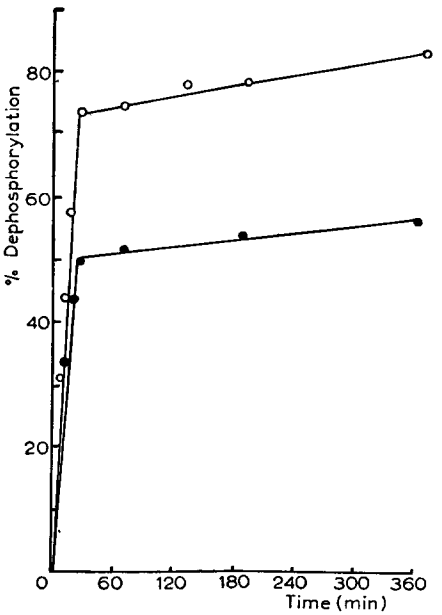

Fig. 3. Dephosphorylation of phosvitin (177.0 μg protein phosphorus) (O—O—O) and lipovitellin (170.0 μg total phosphorus) (●—●—●) by 0.25 *N* barium hydroxide.

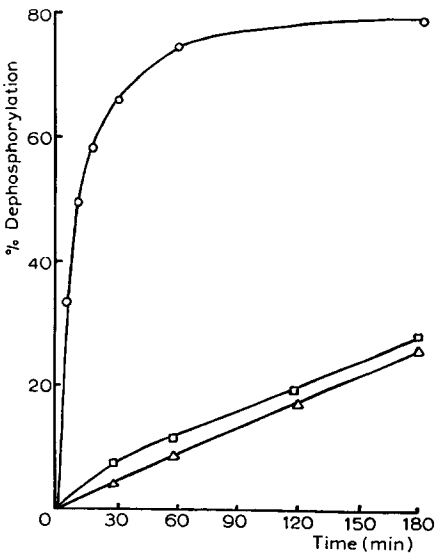

Fig. 4. Dephosphorylation of unfractionated casein (120 μg protein phosphorus) by 0.25 *N* solution of $Ba(OH)_2$ (O—O—O), KOH (□—□—□) and NaOH (△—△—△).

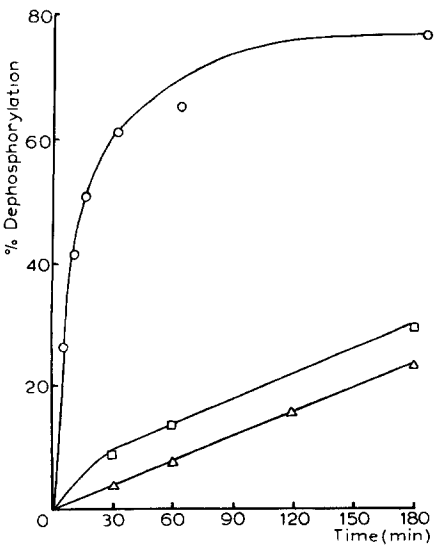

Fig. 5. Dephosphorylation of α-casein (141.0 μg protein phosphorus) by 0.25 *N* solution of $Ba(OH)_2$ (O—O—O), KOH (□—□—□) and NaOH (△—△—△).

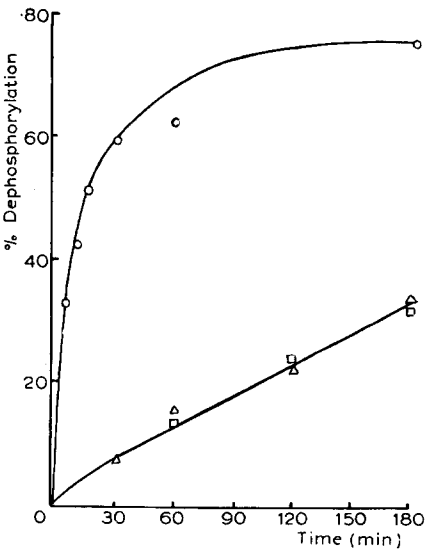

Fig. 6. Dephosphorylation of β-casein (98.5 μg protein phosphorus) by 0.25 *N* solution of $Ba(OH)_2$ (O—O—O), KOH (□—□—□) and NaOH (△—△—△).

took place in a linear fashion throughout the period of study but the rate was considerably slower compared with that observed with barium hydroxide. Incidentally, it may be mentioned that the different fractions of casein are also readily dephosphorylated by phosphoprotein phosphatase[16], though they exhibit marked difference in their behaviour towards non-specific phosphatases[17].

Dephosphorylation of phosphopeptone and phosphoserine. Casein phosphopeptone, prepared according to DAMODARAN AND RAMACHANDRAN[18], was dephosphorylated only to a limited extent (Table IV). Dephosphorylation proceeded at a rapid rate initially and about 45% of the phosphorus was split off within an hour. Maximum dephosphorylation obtained with this substrate was only 65%. *o*-Phosphoserine was completely resistant to dephosphorylation by alkali.

TABLE IV

DEPHOSPHORYLATION OF CASEIN PHOSPHOPEPTONE BY ALKALI

Phosphopeptone concentration: 4 mg (148 μg organic phosphorus) per ml of incubation mixture.

Time of incubation (hours)	*% Dephosphorylation*
1	45
3	45
6	60
24	65

Dephosphorylation of tissue phosphoproteins. Dephosphorylation of the phosphoprotein fraction from liver and mammary gland was investigated, employing barium hydroxide as well as a protein phosphatase preparation from spleen[10] as dephosphorylating agents. The tissues were obtained from a lactating rat, which had received an intraperitoneal injection of ^{32}P-labelled Na_2HPO_4 (150 μC) 2 h prior to being sacrificed. The phosphoprotein fraction was prepared essentially according to the procedure of SCHNEIDER[19] as modified by FRIEDKIN AND LEHNINGER[20]. The acid-insoluble fraction, after exhaustive extraction for lipids, was dried and used directly as substrate. The results, presented in Table V, show that the protein fraction

TABLE V

DEPHOSPHORYLATION OF TISSUE PHOSPHOPROTEIN

SCHNEIDER protein fraction from tissue incubated with alkali (saturated barium hydroxide) and enzyme (spleen protein phosphatase). Radioactivity measured in planchets at infinite thickness with a Geiger tube coupled to a "Panax" counting equipment, according to SCHMIDT AND DAVIDSON[6].

Tissue	*Incubation period (hours)*	*Dephosphorylating agent*	*Activity per 100 mg protein c.p.m.*	*Percentage dephosphorylation**
Liver	3	Alkali	7,660	89
	20	Alkali	8,600	100
	20	Enzyme	6,175	72
Mammary gland	3	Alkali	22,560	78
	20	Alkali	29,070	100
	20	Enzyme	23,860	82

* Dephosphorylation expressed as percentage of phosphorus removed by barium hydroxide in 20 h.

References p. 158.

from these tissues resembles other phosphoproteins in being readily dephosphorylated by alkali and by protein phosphatase. The isolation of phosphoserine from the SCHNEIDER protein fraction of liver has been reported recently by several workers[21,22].

Preparation of dephosphorylated casein

The preparation of alkali-dephosphorylated casein, through the use of NaOH has been described by RIMINGTON[23] and by PLIMMER and co-workers[24,25]: This method has several disadvantages, the chief among which is that the alkali, at the concentration employed, splits up the protein molecule into several components. Results of an essentially similar nature have also been obtained for vitellin by MCFARLANE[2]. It is not quite clear whether the proteolysis observed in these experiments is a direct result of the dephosphorylation reaction or only a non-specific reaction brought about by the alkali. For this reason it was considered of interest to investigate whether alkali has a similar effect on non-phosphorus proteins. The results of such an experiment, presented in Table VI, clearly show that 0.25 *N* sodium hydroxide brings about the liberation of considerable amounts of acid-soluble nitrogen from all the proteins tested. These results suggest that the liberation of inorganic phosphorus and of acid-soluble nitrogen from casein in these experiments are actually two distinct reactions, each proceeding independent of the other.

TABLE VI

ACID-SOLUBLE NITROGEN FORMED FROM SEVERAL PROTEINS BY INCUBATION WITH 0.25 *N* NaOH

2% solutions of the protein in 0.25 *N* NaOH incubated at 37° and the nitrogen estimated in the trichloroacetic acid-soluble fraction.

Protein	*Incubation period (h)*	*% Nitrogen solubilized**
"Unfractionated" casein	3	12.2
	6	15.0
	24	21.2
α-Casein	3	8.0
	6	11.0
	24	18.6
β-Casein	3	4.9
	6	11.9
	24	25.1
Ovalbumin	3	3.3
	6	8.1
	24	19.8
Human blood fibrin	3	11.7
	6	16.2
	24	46.3
Bovine plasma albumin	3	0.2
	6	6.1
	24	14.4
Dephosphorylated casein	3	11.4
	6	15.0
	24	39.6

* Values not corrected for free ammonia present in the digest.

References p. 158.

The observation that barium hydroxide would, at low concentrations, dephosphorylate phosphoproteins (Fig. 1) suggested the use of this reagent as a more specific dephosphorylating agent. Preliminary studies carried out with various concentrations of barium hydroxide indicated that 0.05 *N* alkali brought about maximum dephosphorylation of casein in 20 h, while the liberation of acid-soluble nitrogen with the reagent was reduced to a minimum (6.5% of protein nitrogen). For the preparation of dephosphorylated casein, 5 g of casein in the form of its sodium salt was dissolved in 250 ml of 0.05 *N* barium hydroxide and the solution incubated in a stoppered bottle at 37°. At the end of 20 h, the digest was adjusted to pH 5.5 by the drop-wise addition of acetic acid. The precipitated protein was separated on the centrifuge and washed twice with water. For purification, the protein was dissolved in the minimum amount of ammonia and the solution dialysed in a cellophane bag against several changes of distilled water, in the cold. The protein was reprecipitated as before and was washed several times with water and finally with acetone. The dry product weighing about 2.3 g contained very little phosphorus (0.04%) and had a nitrogen content of 14.8%. The value for nitrogen is somewhat lower than that obtained for enzymically dephosphorylated casein[10], but higher than that reported for NaOH-treated casein (13.8%)[25].

Action of ammonia on casein. Isolation of phosphorus-containing degradation products

The preparation from vitellin of a peptide of high phosphorus content, termed vitellinic acid, by the action of strong ammonia, has been reported by LEVENE AND ALSBERG[26]. Since the phosphorus groupings of casein were resistant to dephosphorylation by ammonia, the possibility of isolating an analogous phosphorus-containing compound from casein was considered worth investigating. Unfractionated casein as well as α-casein were used as starting material in these investigations.

The protein, in the form of its sodium salt, was dissolved in 20 volumes of 2.5 *N* ammonia and the solution incubated in a stoppered bottle at 37°. At the end of 48 h, the digest was aerated to remove ammonia and the pH then adjusted to 4.8 by the addition of acetic acid. The precipitate that separated at this pH was collected by filtration and repeatedly washed with water. For purification it was dissolved in dilute alkali and reprecipitated by the addition of acid to pH 4.8. After several washings with water the protein was dried by treatment with acetone.

The filtrate from the protein was concentrated *in vacuo* to dryness. The gelatinous mass left behind at the end of distillation was taken up in about 10 to 15 ml of water, stirred well and filtered. The clear filtrate, pale yellow in colour, was treated with 10 to 15 volumes of ethanol to precipitate the proteoses. After keeping overnight in the ice chest, the precipitate was collected by centrifugation. For further purification it was dissolved in water, filtered and reprecipitated with ethanol. The precipitate was washed several times with ethanol and dried *in vacuo* over sulphuric acid.The final product was obtained as flakes, which on grinding in a mortar yielded a fine powder.

The filtrate left after the separation of the proteose was diluted with an equal volume of water to bring down the concentration of alcohol to 50%. Sufficient quantity of a 25% (w/v) solution of barium acetate was next added and the solution left overnight in the ice chest. The precipitated barium salt was separated, dissolved in water, and reprecipitated with 50% ethanol. The product was washed with several

References p. 158.

portions of 50% (v/v) ethanol and dried by washing with ethanol, followed by ether. Starting from 10 g of casein, about 150 mg of proteose and 50 mg of the peptone were obtained. The nitrogen and the phosphorus content of the various fractions are given in Table VII.

The phosphopeptone fraction, after removal of barium by the quantitative addition of dilute sulphuric acid, was examined by paper electrophoresis in $M/10$ acetic acid. The peptone appeared to be homogeneous since it gave only a single ninhydrin-positive phosphorus-containing band. After complete hydrolysis of the compound with 6 N hydrochloric acid, the following amino acids were detected in the hydrolysate, as revealed by ascending paper chromatography in n-butanol–glacial acetic acid–water (4:1:5) solvent system: phosphoserine, aspartic acid, glutamic acid, glycine, alanine, serine, phenylalanine, valine, and leucine (and/or isoleucine).

TABLE VII

CHARACTERIZATION OF PRODUCTS OBTAINED BY THE ACTION OF AMMONIA ON CASEIN

Protein	*Fraction*	*Nitrogen** %	*Phosphorus** %	*N/P ratio atomic*
Unfractionated casein	Protein	13.64	0.51	59.2
	Proteose	12.1	1.23	21.8
	Peptone	5.4	4.49	2.7
α-Casein	Protein	12.03	0.56	47.5
	Proteose	11.24	1.60	15.6
	Peptone	5.17	3.83	3.0

* Values not corrected for moisture.

DISCUSSION

The results of the present study definitely show that the dephosphorylating action of alkali on phosphoproteins is governed by several factors. While the pH of the medium has a definite influence on the reaction it is not, however, the sole factor determining the extent of dephosphorylation. The marked difference exhibited by various alkalis in their dephosphorylating action can only be explained on the assumption that the nature of the cation present in them profoundly influences the rate of the reaction. The results presented in Table II amply justify such an assumption and point to the conclusion, that the alkali dephosphorylation of phosphoproteins is a metal-catalysed reaction. A similar finding has been reported recently by BAMANN, TRAPMANN, AND SCHEUGRAT[27], who investigated the effect of certain rare-earth metal ions on the dephosphorylation of phosphoproteins in alkaline medium. In their experiments, cerium was observed to accelerate the dephosphorylation of the phosphorylated derivatives of serine and threonine, while lanthanum had a similar effect on the dephosphorylation of casein and vitellin, though with the latter substrates the effect was manifested only at high temperatures.

Dephosphorylation studies carried out with various phosphoproteins indicate that the phosphorus groupings in most of them exhibit unequal susceptibilities in their dephosphorylation behaviour. This is quite apparent in the case of ovalbumin and phosvitin. With the former, about 50% of its phosphorus is found to be extremely

References p. 158.

labile towards alkali, while the rest of it is removed only at a slow rate. Similarly in the case of phosvitin, about 75% of its phosphorus is much more readily available for dephosphorylation compared to the rest. There may be several explanations to account for this phenomenon. The phosphorus groupings resistant to dephosphorylation may be sterically protected, though it must be mentioned that in casein phosphopeptone, which is obtained after an extensive proteolytic degradation of casein, about 35% of its phosphorus remains unavailable for dephosphorylation (Table IV). The presence of different types of phosphorus linkage in a protein may also conceivably cause such results, since phosphorus, linked in different ways, may not be equally susceptible to dephosphorylation. This explanation may hold good in the case of ovalbumin and casein, where the existence of more than one type of phosphorus linkage has been demonstrated[17], but not for phosvitin, which contains mainly monoesterified phosphate[7]. Lastly, the amino acid configuration around the phosphorylated amino acid may also influence its dephosphorylation to an appreciable extent. This is well brought out by the recent experiments of FLAVIN[15], who showed that the two phosphoserine units in ovalbumin, which exhibit such marked difference in their dephosphorylation behaviour, are present in entirely different amino acid sequences.

It is of interest to note that phosphoserine, in the free state, is quite stable towards alkali. This suggests that the properties of an amino acid may change appreciably when incorporated into a peptide. In this connection it is pertinent to recall the experiment of NICOLET AND SHINN[28] on the dephosphorylation of phosphoserine and its derivatives by alkali. These workers found that phosphoserine was completely resistant to the action of 0.25 *N* NaOH and that substitution in the carboxyl group of the amino acid made the phosphorus group somewhat labile, while masking of both the amino and carboxyl groups of the amino acid increased the alkali lability of its phosphate still further.

It is well known that the phosphorus groupings in casein are concentrated in relatively small parts of the molecule and could be readily isolated by enzymic hydrolysis of the protein[18,29,30]. The results obtained in the present study show that the preparation of such phosphopeptones may also be brought about by chemical methods. It is of interest to note that the peptone obtained by the action of ammonia on casein contains the amino acids, serine, glutamic acid, and isoleucine, which also occur in the preparations obtained by other workers. However, while the phosphopeptone preparations obtained by enzymic methods appear to be made up of a multiplicity of peptides[31], there are indications that the peptone obtained in the present investigation is composed of a single component. A detailed study of the composition of the peptone has been undertaken and will form the subject of a future communication.

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References p. 158.

SUMMARY

1. It has been observed that alkalis differ widely in their ability to dephosphorylate phosphoproteins. The hydroxides of calcium, strontium and barium were thus much more effective as dephosphorylating agents, compared with those of sodium and potassium. Ammonia, which was ineffective by itself, readily dephosphorylated phosvitin in the presence of certain metal ions. It is concluded from these studies that the dephosphorylation of phosphoproteins is catalysed by metal ion and that the dephosphorylating action of an alkali on phosphoprotein is influenced to a large extent by the nature of the cation present in it.

2. Dephosphorylation studies carried out on different phosphoprotein substrates indicate the presence in these of phosphate groupings resistant to dephosphorylation. Several explanations have been offered to account for this observation.

3. Phosphoprotein fraction obtained from tissues by SCHNEIDER's procedure has been shown to resemble other phosphoproteins in its dephosphorylation behaviour towards alkali and enzyme.

4. An improved procedure for the preparation of alkali-dephosphorylated casein, which involves the use of dilute barium hydroxide as dephosphorylating agent, and a chemical method for the preparation of a phosphopeptone from casein, have been described.

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