The Chemical and Immunological Properties of Phosphorylated Proteins

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The natural phosphoproteins such as casein and ovovitellin offer much of interest, chemically and immunologically, and many investigators have studied both these and artificially phosphorylated proteins, to determine how the phosphate groups are linked to the protein molecule; comparisons of the chemical properties of both types of protein have also been made by several workers. Phosphoryl chloride was used as phosphorylating agent by Bechhold (1901), Neuberg & Pollak (1910) and Neuberg & Oertel (1914). These methods were modified by Rimington (1927), who treated the ice-cold protein solution with phosphoryl chloride in carbon tetrachloride, adding alkali at intervals to keep the solution faintly alkaline. Rimington described the preparation and properties of phosphorylated proteins prepared from casein, denatured serum globulin and dephosphorized casein.

A comprehensive chemical and immunological investigation of phosphorylated egg albumin was made by Heidelberger, Davis & Treffers (1941). They found that phosphorylation, which was accompanied by some denaturation, effected a profound change in the serological reactions of the egg albumin. Immunological investigations have also been made on phosphorylated casein (Ecker & Simon, 1928) and casein, dephosphorized casein and rephosphorized casein (Kesztyus & Kocsis, 1942).

Our own investigations with phosphorylated proteins were started with two main objects. It was hoped that the introduction into the protein molecule of phosphate groups containing radioactive P would yield labelled antigens which could be used for serological investigations, e.g. for studies on the fate of injected protein antigens and for quantitative studies on the precipitin reaction. It was also intended to study the nature of the linkage between the phosphate group and the protein in phosphoproteins and phosphorylated proteins, by immunochemical and isotopic tracer methods. Some of the results of these investigations are given in the present paper.

EXPERIMENTAL

Phosphorylation and dialysis. The method used was essentially that described by Rimington (1927). Freshly distilled POCl_3 , dissolved in about 8 vol. of carbon tetra-

chloride, was added slowly to the protein solution cooled in ice water; varying amounts of POCl_3 were used, usually ranging from 0.1 to 0.5 g./g. of protein. The mixture was stirred continuously and maintained at an alkaline pH (pink to phenolphthalein added as an internal indicator) by the addition of N- or 4N- NaOH at intervals during the addition of the POCl_3 and for some time afterwards. When no more NaOH was required, the aqueous layer was dialyzed at about 5° in cellophan sacs against 0.9% (w/v) NaCl (to which a little toluene had been added) until free from inorganic phosphate. In some of the earlier experiments distilled water was used as the dialyzing fluid. To determine the extent of the phosphorylation, total P and total N determinations were made on the original solution and on samples of the phosphorylated proteins precipitated from the solution in various ways. The results are usually expressed as a ratio (wt. of P/wt. of N).

Precipitation of the phosphorylated proteins. In most of the experiments the phosphorylated protein was precipitated from solution by adjusting the pH to 4.4-4.6 with 0.1 N-HCl, using external indicators; the best results were obtained by using 2% (v/v) acetic acid for final adjustment of the pH to give maximal precipitation. Precipitation was least satisfactory and sometimes impossible with phosphorylated proteins of low P/N ratios. In several experiments the proteins were precipitated by the addition of 4 vol. of a 10% (w/v) solution of trichloroacetic acid (subsequently referred to as TCA). When these precipitates were washed with ethanol or acetone to remove TCA, some protein dissolved, but this was precipitated again by the addition of N- NaOH and a few drops of conc. MgCl_2 solution. The unwashed precipitates obtained with TCA, when dried over CaCl_2 , had a horny texture. In other experiments the phosphorylated protein was precipitated with 6 vol. of ethanol, since the precipitates obtained with TCA gave lower P/N ratios than did those obtained with HCl; it was thought possible that some of the less firmly attached phosphate groups had been removed from the protein by treatment with TCA. The P/N ratios of ethanol-precipitated phosphorylated proteins were almost always the same as those for the HCl-precipitated product from the same solution, where this solution was free from inorganic phosphate. Where, however, the solution contained inorganic phosphate, the ethanol-precipitated proteins gave a relatively high P/N ratio, and tests made with ^{32}P containing inorganic phosphate showed that this was due to the precipitation of appreciable amounts of inorganic phosphate by the ethanol. The results obtained with ethanol-precipitated products have therefore been discarded, except where they refer to precipitates obtained from solutions free from inorganic phosphate.

P determinations. These were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Preparation of phosphorylated proteins for immunization

'High P' proteins. Sterile human serum (110 ml.) was phosphorylated in two equal batches, using a POCl_3 /protein ratio of 0.5/1. After dialysis, the phosphorylated proteins were precipitated by acid (N-HCl and 2% acetic acid) and redissolved in sterile 0.9% NaCl with the addition of a little N-NaOH. Phenol (final concentration 0.25%) was also added. This solution had a protein content of 5.1% (w/v) and a P/N ratio of 0.14.

'Low P' proteins. Sterile human serum (165 ml.) was phosphorylated, dialyzed, precipitated and redissolved as above. The final solution contained 7.25% protein (P/N ratio 0.14) and 0.25% phenol. A mixture of this solution and 0.25 vol. of 0.75M- NaHCO_3 - H_2CO_3 buffer (pH 7.5) was placed in a stoppered bottle, the air in the bottle displaced by CO_2 and the bottle sealed and kept at 39° for 139 hr. The solution was then dialyzed against cold 0.9% NaCl to remove any free phosphate which had been split off at 39°, and 0.25% of phenol added. This solution contained 5.2% protein with a P/N ratio of 0.02.

Preparation of antigens for precipitin tests. Rabbit plasma and chicken serum were phosphorylated using a POCl_3 /protein ratio of 0.5/1. The phosphorylated rabbit plasma had a P/N ratio of 0.17; some of this solution was kept at 37° with an equal volume of 0.75M- NaHCO_3 - H_2CO_3 buffer (pH 7.5) for 4 days, followed by dialysis ('low P' rabbit proteins). In an attempt to obtain a phosphorylated antigen having a higher P/N ratio, rabbit serum was phosphorylated using a POCl_3 /protein ratio of 2.5/1, but the product had the same P/N value (0.17) as the phosphorylated rabbit plasma preparation described above. Gelatin solutions (0.25 and 0.5%) were treated with different amounts of POCl_3 (POCl_3 /protein ratios of 0.5 and 2.5/1 respectively). The amount of protein present after dialysis was, however, very small, and in both cases there was insufficient material for accurate P determinations.

Immunization. The 'high P' and 'low P' phosphorylated human serum proteins were each injected into a group of five rabbits. Two rabbits of each group received an initial intravenous injection of 4.5 or 5 ml. of the solution, followed by five or six intraperitoneal injections of 5 ml. (rising gradually to 10 ml.) at intervals of 7–10 days. Sera from these rabbits were tested after the third and subsequent injections. The remaining three rabbits of each group received three courses of intraperitoneal injections; each course consisted of four or five injections (each of 1.8 or 2.0 ml. of 5% protein solution) at intervals of 2–4 days, with an interval of 8–12 days between the courses. Sera for precipitin tests were collected after the second and third courses.

Precipitin tests. The tests were made as described previously (Hopkins & Wormall, 1933). The concentration of antigen in the Tables refers to dilution (with 0.9% NaCl) of a solution of the antigen containing approx. 5% protein.

RESULTS

Phosphorylation of 'dephosphorized casein', serum globulin and serum albumin

'Dephosphorized casein' was prepared from 'soluble casein' (British Drug Houses Ltd.) by the action of 0.25N-NaOH (Plimmer & Bayliss, 1906; Rimington & Kay, 1926), the

product being purified by two precipitations. The globulin and albumin were prepared from human serum by half and full saturation with $(\text{NH}_4)_2\text{SO}_4$; they were purified by repeated precipitation, and were finally dialyzed against 0.9% NaCl until free from $(\text{NH}_4)_2\text{SO}_4$. Sufficient 0.9% NaCl was added to each solution to give a protein concentration of 4.8% (w/v).

Each solution (20.8 ml. of dephosphorylated casein, globulin or albumin) was phosphorylated with 0.5 g. of POCl_3 (i.e. a POCl_3 /protein ratio of 0.5/1), as described above. During the course of phosphorylation the globulin and dephosphorized casein solutions became gelatinous, and adequate stirring was difficult; 0.9% NaCl, equal to about 0.25 of the total volume of the solution, was therefore added before the phosphorylation had been completed. The albumin solution was similarly diluted before phosphorylation. After dialysis the phosphorylated proteins were repeatedly precipitated with HCl, the precipitates being washed with distilled water to which a few drops of acetic acid had been added. P/N ratios, determined on the precipitates obtained in the 4th, 5th and 6th precipitations are given in Table 1.

The results showed that, under these conditions, phosphorylation of serum albumin, serum globulin and dephosphorized casein with POCl_3 readily effects the introduction of one P atom for every 33–38 N atoms in the protein molecule; this represents a P content of 1.0–1.07% for the phosphorylated proteins, calculated on the basis of 16% N in the protein. It is interesting to note that approximately the same amount of P was introduced into three different types of protein. The results also show that after three precipitations of these phosphorylated proteins with HCl, further acid precipitation yielded products of approximately constant P content.

Table 1. P contents of 'rephosphorized' casein and phosphorylated human serum albumin and globulin

Precipitation	P/N (weight ratios)		
	Phosphorylated albumin	Phosphorylated globulin	Rephosphorized casein
4th	0.062	0.067	0.063
5th	0.059	0.070	0.062
6th	0.057	0.065	0.060

Conditions of preparation of phosphorylated human serum proteins

Effect of varying the POCl_3 /protein ratios and other experimental conditions. Since it was our intention to introduce ^{32}P into proteins for immunological investigations several experiments have been carried out to determine the amount of P which enters the protein when it is treated with amounts of POCl_3 considerably less than those used by Rimington (1927) and other investigators. In a typical experiment, three samples, each of 14.3 ml. of pooled human serum and containing 1 g. of protein, were treated with 0.5, 0.3 and 0.1 g. of POCl_3 respectively. The P/N ratios of the dialyzed solutions and of the acid-precipitated products from the 1st, 2nd and 3rd precipitations showed that with the smaller amounts of POCl_3 less P was introduced into the

Table 2. Comparison of P/N ratios of human serum proteins phosphorylated under varying conditions

Material	Protein content (%)	Ratio POCl ₃ /protein	Dialyzed solution	P/N ratio		
				HCl precipitate		
				1st	2nd	3rd
Human serum	7.25	0.5	0.104	0.111	0.100	0.101
		0.3	0.078	0.083	0.074	0.071
		0.1	0.030	0.033	0.031	0.033
Freeze-dried human serum	7.0	0.3	0.075	—	—	—
	12.5	0.3	0.095	—	—	—

protein (Table 2). With POCl₃/protein ratios of 0.5/1, 0.3/1 and 0.1/1, the resulting phosphorylated proteins contained 1.62, 1.14 and 0.53 % of P respectively, calculated on a 16 % N content for the protein. This would correspond respectively to the introduction of about 52, 37 and 17 P atoms into each protein molecule, assuming a mean mol. wt. of 10⁵ for the serum proteins.

Phosphorylation of a concentrated solution of human serum proteins. It was thought possible that less POCl₃ would be lost by hydrolysis if the solution to be phosphorylated had a higher protein content. This point was tested using a concentrated solution of serum proteins and a relatively low POCl₃/protein ratio. Samples of freeze-dried human serum (kindly presented by Dr R. R. Race) were dissolved in water to give 12.5 and 7 % protein solutions. These solutions were phosphorylated with sufficient POCl₃ to give a POCl₃/protein ratio of 0.3/1 and then dialyzed. The results recorded in the lower part of Table 2 showed that a saving of POCl₃ could be effected by the use of the more concentrated protein solution, but this method was not used in later experiments since it gave a greater amount of insoluble material than did phosphorylation of normal serum.

Stability of the phosphoryl group attached to proteins

Preliminary investigations had suggested that loss of phosphoryl groups occurred when the phosphorylated proteins were kept at 37°, even at or near neutrality, and the following experiments were therefore carried out to determine to what extent

this loss might occur under physiological conditions, e.g. after injection into the animal body.

Loss of P from phosphorylated human serum proteins, 'rephosphorized' casein and casein at 37°. Solutions of phosphorylated human serum proteins, casein, and in one case rephosphorized casein (all phosphorylated with a POCl₃/protein ratio of 0.5/1 and precipitated by HCl), were treated with equal volumes of either 0.75M-NaHCO₃-H₂CO₃ buffer (pH 7.5) or veronal buffer (pH 6.8) and the mixtures sealed in bottles with a little toluene, and kept at 37°. Samples were taken at intervals and the proteins precipitated by TCA. The results (Table 3) showed that the phosphorylated human serum proteins had lost appreciable amounts of combined P when the solutions (at pH 6.8 or 7.5) were kept at 37°; at least one fifth of the combined P in 24 hr. and well over half in 6 days. Somewhat similar results were obtained in an experiment with rephosphorized casein (at pH 6.8). The P of casein is, however, much less labile under these conditions, and the loss in 6 days was usually less than 10 %. In another experiment the incubation at pH 7.5 was continued for 20 days, with results similar to those recorded in Table 3; analysis of the TCA-precipitated proteins showed that the phosphorylated serum proteins had lost about half their combined P during the incubation, whereas casein had lost little or none.

Table 3. P content of phosphorylated proteins kept at 37° and pH 6.8 or 7.5

Exp.	pH	Duration of incubation (hr.)	P/N ratio of TCA-precipitated proteins		
			Phosphorylated human serum proteins	Rephosphorized casein	Casein*
1	7.5	0	0.051	—	0.052
		24	0.041	—	0.048
		72	0.027	—	0.047
		147	0.024	—	—
	6.8	0	0.067	0.042	0.048
		24	0.049	0.039	0.047
		72	0.027	0.025	0.046
		147	0.022	0.021	—
	7.5	0	0.041	—	0.042
		142	0.018	—	0.039
		306	0.020	—	0.036

* The casein separated out from both buffer mixtures during the incubation, but samples were taken from the suspension after shaking.

Loss at 37° of P from phosphorylated serum proteins prepared with different POCl₃/protein ratios. Different preparations of phosphorylated serum proteins showed differences in the rate of loss of combined P when the solutions were kept at 37° and it was thought that there might be qualitative differences in this respect between the phosphate groups most readily attached to the protein (i.e. by the use of lesser quantities of POCl₃) and those attached by more drastic phosphorylation.

Human serum (20 ml.) was phosphorylated with 0.14 g. POCl₃ (i.e. a POCl₃/protein ratio of 0.1/1). The product was dialyzed against distilled water for 5 hr., and then against 0.9% NaCl until free from inorganic phosphate. The solution was brought to pH 7.5 with 0.2N-NaOH and added to an equal volume of 0.75M-NaHCO₃-H₂CO₃ buffer. Toluene was added and the mixture was kept in a sealed tube at 37°, samples being withdrawn after 141 and 260 hr. for precipitation of the proteins with TCA.

The results (Table 4) showed that even with the 'lightly phosphorylated' proteins there was fairly rapid loss of P when the solution was kept at 37°.

Table 4. *Loss at 37° and pH 7.5 of P from phosphorylated human serum proteins prepared with a POCl₃/protein ratio of 0.1/1*

Duration of incubation (hr.)	Type of precipitation*	P/N ratio
0	(Dialyzed liquid)	0.042
	HCl + acetone	0.040
	TCA	0.021
141	TCA	0.005
260	TCA	0.004

* The precipitates obtained with TCA were washed with ethanol and acetone.

A further experiment was made to cover a greater range of POCl₃/protein ratios, viz. 0.025-0.05/1. All these resulting phosphorylated human serum proteins, with P/N ratios ranging from 0.017 to 0.095, lost 80% of their combined P when the solutions were kept at 37° for 142 hr. (Table 5). The possibility

Table 5. *Loss of P at 37° and pH 7.5 from phosphorylated human serum proteins prepared with different POCl₃/protein ratios*

POCl ₃ /protein ratio used in the preparation	P/N ratio	
	Dialyzed* solution	After 142 hr. at 37° and subsequent dialysis*
0.025	0.017	0.005
0.05	0.024	0.005
0.10	0.038	0.008
0.20	0.055	0.010
0.30	0.076	0.015
0.50	0.095	0.023

* Dialysis at 4-6°.

that some of the phosphorylated protein was split up into smaller molecules of P-containing organic substances during the incubation, and that these smaller molecules subsequently passed through the dialyzing sac, was not explored.

Immunization with phosphorylated serum proteins

Two different antigens were used for immunization in the hope that antisera of differing serological specificity might be obtained. The 'high P' phosphorylated serum protein was prepared by the action of the usual great excess of POCl₃ on human serum proteins, and the 'low P' protein was obtained by incubating a solution of the 'high P' proteins at 39° and pH 7.5, with subsequent dialysis to remove free phosphate; incubation for 6 days removed about 6/7 of the combined P of the 'high P' phosphorylated proteins.

Precipitin tests carried out with the sera of the injected rabbits over a long period of immunization showed that all the rabbits had produced antibodies capable of reacting strongly with the antigens used for the injections (phosphorylated human serum proteins), with no marked difference between the results with the 'high P' and those with the 'low P' proteins. The antisera also gave strong or very strong precipitin reactions with untreated human serum, showing that phosphorylation had not destroyed the species specificity of the serum proteins. In a few cases only, the antisera reacted slightly with phosphorylated rabbit serum proteins, but no precipitate was obtained with phosphorylated chicken serum proteins or with phosphorylated gelatin. The results of some typical precipitin tests are given in Table 6.

DISCUSSION

The phosphorylated proteins obtained by the action of POCl₃ on serum proteins are readily precipitated by dilute acid (at pH 4-5 for the strongly phosphorylated proteins). In many other respects also, phosphorylation effects a distinct change in properties. With a moderate excess of phosphoryl chloride, the amount of P introduced into the proteins under the conditions of our experiments may correspond to as much as 50 phosphoryl groups/mol. of protein, assuming a mean mol. wt. of 10⁵ for these proteins. Heidelberger *et al.* (1941), in similar investigations, found that crystalline egg albumin could take up 20-30 phosphoryl groups/mol. on the basis of a mol. wt. of 36,000 for egg albumin, and they noted 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'.

Table 6. *Precipitin reactions with antisera to phosphorylated human serum proteins*

Antigen	Dilution of antigen	Antisera to phosphorylated human serum proteins			
		'Low P'		'High P'	
		Rabbit no. 461	Rabbit no. 463	Rabbit no. 480	Rabbit no. 482
Human serum	1:20	+	±	±	++
	1:100	++±	++±	+	++±
	1:500	++	±	±	+
	1:2500	±	tr	tr	+
Phosphorylated human serum proteins	'Low P'	1:20	±	tr	.
		1:100	++±	+	.
		1:500	±	tr	.
		1:2500	±	tr	.
	'High P'	1:20	+	tr	±
		1:100	++±	+	++±
		1:500	±	±	++±
		1:2500	±	tr	+
Phosphorylated rabbit serum proteins	(a)*	1:20	-	-	-
		1:100	-	-	-
		1:500	-	-	±
		1:2500	-	-	-
	(b)*	1:20	-	-	-
		1:100	-	-	f.tr
		1:500	-	-	tr
		1:2500	-	-	±

* The phosphorylated rabbit serum preparations (a) and (b) were obtained by the use of a POCl_3 /protein ratio of 0.5/1 and 2.5/1 respectively; both products were dialyzed free from inorganic phosphate.

Precipitin readings: - (no precipitate); f.tr (faint trace); tr (trace); ±, +, ++, etc. in increasing degrees of precipitation.

In most of the experiments described in this paper we have used phosphorylated mixed serum proteins, the chief object being to prepare antigenic proteins containing ^{32}P , and a mixture of the phosphorylated serum globulins and albumins seemed most suitable for many of the serological tests. For some of the experiments reported here, however, and for others to be described later, purified and less complex mixtures of phosphorylated proteins have been used. Another difference between our experiments and those of Heidelberger *et al.* (1941) is that in nearly all cases we have used considerably less phosphoryl chloride than was used by them, or by Rimington (1927). Our object was to ensure minimum loss of valuable ^{32}P in parallel experiments with 'radio' POCl_3 . Even with this smaller amount of phosphoryl chloride (0.5 g./g. of protein as compared with the 2-3 g./g. of protein used by Heidelberger *et al.* and by Rimington in most of their experiments) the proteins were phosphorylated to a comparable degree: for example, 1-1.5% of P, as compared with 1.52% for heavily phosphorylated egg albumin (Heidelberger *et al.* 1941), and 0.71% for phosphorylated denatured serum globulin (Rimington, 1927). Furthermore, in one of our experiments it was found that a fivefold increase in the amount of phosphoryl chloride used did not lead to any significant increase in the amount of P attached to rabbit serum proteins.

The amount of P introduced into the serum proteins varied somewhat from one preparation to another, and the reason for this variation is not known. Freshly distilled POCl_3 was used in practically all our experiments, and the technique of phosphorylation was kept as constant as possible. Different samples of human serum may differ in their capacity to react with POCl_3 , but it is difficult to understand why, for example, a P/N ratio of 0.06-0.07 was obtained for the phosphorylated human serum albumin and globulin in one experiment (Table 1), whereas most subsequent experiments with serum proteins, and involving the use of the same relative amount of phosphoryl chloride, gave products with a P/N ratio of approximately 0.10. Similar variations were also noted by Heidelberger *et al.* (1941): one of their phosphorylated egg albumin preparations contained 2.71% of P and had a P/N ratio of 0.18, as against P/N ratios of 0.104-0.115 for other preparations obtained by the same method of phosphorylation. It seems probable, as they suggest, that part of the P of phosphorylated proteins with a high P/N ratio is in a very labile form, and that variable amounts are split off when the experimental technique for isolating the phosphorylated protein is varied even slightly. We, like they, have found that this lability is very marked at 37°.

Of the various protein precipitants which we have used for the separation of the phosphorylated proteins, dilute acid (HCl, followed by final adjustment of the pH with acetic acid) has proved most satisfactory. Trichloroacetic acid was less satisfactory, and it is possible that the high acidity caused by this reagent (about pH 0.6 in the experiments referred to in Table 3) may have resulted in appreciable removal of P from the phosphorylated proteins. Where a solution of phosphorylated protein was required for immunological purposes, purification was effected by dialysis, followed by repeated acid precipitation where the preparation was required for injection.

The results of the immunological experiments showed that the antibodies produced by the injection of phosphorylated human serum proteins were not specific for the phosphoryl groups of the protein, for the antisera obtained gave little or no reaction with phosphorylated rabbit or chicken serum proteins. This result was rather surprising, for the introduction of an appreciable number of polar groups into the molecule of an antigenic protein usually leads to the acquisition of a new serological specificity characteristic for the introduced groupings. It is probable, however, that the phosphorylated human serum proteins rapidly lost most of their combined P after injection into rabbits; there was certainly no lack of antigenic power in the injected material, since powerful antibodies against the injected antigen (phosphorylated human serum proteins) were formed. This view is supported by the *in vitro* lability of the combined phosphate groups at 37° and pH 7.5 (Tables 3-5); incubation for several days resulted in a very marked decrease, sometimes as much as 80 %, in the combined P of the proteins precipitable by trichloroacetic acid or those freed from dialyzable P-containing compounds.

If the injected phosphorylated proteins rapidly lost their combined P, as the experiments described in this paper and in the related investigations with ³²P-containing phosphorylated proteins (Banks, Bourns, Dewey, Francis, Tupper & Wormall, 1948) suggest, the failure of the injected rabbits to produce antibodies to phosphorylated heterologous protein, and the production of antibodies capable of reacting with untreated human serum proteins, can readily be explained. It seems probable that our phosphorylated human serum protein preparations differed considerably, in their *in vivo* stability, from the phosphorylated egg albumin of Heidelberger *et al.* (1941), for the antisera produced by the injection of the latter antigen reacted only slightly with unchanged egg albumin. Heidelberger *et al.* obtained specific antibodies against phosphorylated egg albumin, but they observed that the new specificity conferred on egg albumin by the introduction of phosphoryl groups is not entirely a haptene specificity in the sense defined by Landsteiner (1936).

The possibility of an enzymic removal of P from phosphorylated proteins, either by an enzyme present in our preparations of phosphorylated human serum proteins or in rabbit blood, cannot be excluded, but this possibility has

not as yet been investigated. We intend, however, to study the *in vivo* stability of phosphorylated human serum proteins, phosphorylated egg albumin and other phosphorylated proteins. It seems probable from the evidence already available that phosphorylated human serum proteins may not be the most suitable proteins for our investigations with ³²P-containing protein antigens. For some of these purposes vitellin is more satisfactory (Francis & Wormall, 1948), but unfortunately it is almost insoluble in water when freed from firmly combined phosphatide.

The action of phosphoryl chloride on proteins may concern several groups of the protein, e.g. the hydroxy groups of serine and threonine, and possibly the phenol group of tyrosine, the free amino group of lysine, and the guanidine group of arginine. Some of the attached phosphoryl groupings will, of course, be less firmly attached than others, and the existence of different P-protein linkages might readily explain the considerable variations in the rate with which P is removed from phosphorylated proteins. It is difficult to understand, however, why this process is so rapid, particularly at 37°, in solutions kept at pH 7.5, for some phosphorylated amino-acids are quite stable. Plimmer (1941), for example, found that the phosphoric esters of tyrosine, hydroxyproline, threonine, serine and isoserine were all stable to 0.5N- or even N-NaOH at 37°. It is our intention to study this problem further, with the aid of serological methods.

SUMMARY

1. Human serum proteins have been phosphorylated by phosphoryl chloride, and some of the chemical and immunological properties of the products have been studied.
2. These phosphorylated proteins lost appreciable amounts of their combined P when they were kept in solution at 37° and pH 7.5 or 6.8.
3. Immunization of rabbits with phosphorylated human serum proteins produced antibodies which reacted strongly with the injected proteins and also with untreated human serum proteins.
4. These antisera gave little or no precipitation with phosphorylated rabbit serum proteins or phosphorylated chicken serum proteins. No evidence, therefore, was obtained of the formation of antibodies specific for the phosphoryl group of phosphorylated proteins. It is probable, however, that the phosphorylated serum proteins rapidly lost a considerable fraction of their combined P after injection into the rabbits.

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POSTSCRIPT. Since this paper was in the press we have seen the very valuable account of the chemical and immunological properties of phos-

phorylated crystalline horse serum albumin given by Mayer & Heidelberger (1946). We hope to make full reference to this work later.

REFERENCE. Mayer, M. & Heidelberger, M. (1946). *J. Amer. chem. Soc.* **68**, 18.

Studies in the Biochemistry of Micro-organisms

77. A SURVEY OF FUNGAL METABOLISM OF INORGANIC SULPHATES

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Clutterbuck, Mukhopadhyay, Oxford & Raistrick (1940), in a communication from this laboratory, described the results of a quantitative survey of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0.5 g. KCl/l. as the sole source of chlorine.

The results are now given of a parallel quantitative survey of the sulphur metabolism of 115 species or strains of moulds grown on the same medium containing 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /l. as the sole source of sulphur.

EXPERIMENTAL

Cultural conditions

The medium used throughout this investigation was the well-known Czapek-Dox solution of the following composition: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l. All the constituents of the medium were of analytical reagent purity. Hence the sole source of sulphur was present as the sulphates of magnesium and iron, and amounted to 22.9 mg./flask (6.6 mg./100 ml.) at the time of sowing. This medium (350 ml.) was accurately measured into each of a number of 1 l. conical flasks plugged with cotton wool, and sterilized by steaming for 0.5 hr. on each of 3 consecutive days. The flasks were then inoculated with a pure culture of the chosen species of mould, and incubated at 24° for the requisite period of days.

The sulphate content of duplicate uninoculated flasks from each batch was determined by the method described later. The inoculated flasks were treated as follows. The mycelium from each species was separated quantitatively by filtration through a Buchner funnel, and was thoroughly washed with warm water. It was then dried to constant

weight *in vacuo*, weighed, ground and stored in a closely stoppered bottle prior to examination. The culture filtrate and washings were made up to a total volume of 500 ml., and analyzed by the methods described in the next section.

Analytical methods

I. Sulphur as inorganic sulphate in culture filtrate

The concentration of inorganic sulphate present in the uninoculated medium is quite low (equiv. to 22.9 mg. S/350 ml., or 4.58 mg./100 ml. after dilution to 500 ml.). The medium also contains relatively considerable amounts of nitrate, phosphate and chloride. Further, the culture filtrate, resulting from the growth of different moulds may contain metabolic products of complex and unknown nature and may vary from almost colourless to deeply coloured. After a considerable amount of exploratory work, details of which will be published elsewhere, on a number of published methods the one finally chosen as being the most generally useful for our purpose was that based on Øllgaard's (1934) method. This method involves the precipitation of inorganic sulphate with benzidine in acetone and titration of the precipitate, after liberation of the sulphate with a little NaOH, with a standard BaCl_2 solution using sodium rhodizonate as indicator. Using this method we found that, in solutions containing 0.3 mg. of sulphate S/10 ml. portion, a precision of $\pm 1\%$ in duplicates can in general be assured, and of $\pm 5\%$ with certainty.

Reagents

Trichloroacetic acid. 20% (w/v) solution in water.

Benzidine. 1% solution in pure acetone, freshly prepared each day.

Sodium hydroxide. N-NaOH, in water.

Ethanollic magnesia mixture. NH_4Cl (6 g.) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (6 g.) in water (100 ml.) and made up to 1 l. with absolute ethanol.