The Immunological and Serological Properties of Phosvitin

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In view of the pronounced influence of polar groups in the molecule in determining the specificity of protein antigens, it might be expected that the phosphate groups in natural and artificial phosphoproteins would be largely responsible for the immunological specificity of these proteins. Investigation of their serological properties might therefore yield useful information about the structure, and the biosynthesis, of natural phosphoproteins.

Heidelberger, Davis & Treffers (1941) have investigated the immunological properties of artificially phosphorylated ovalbumin, and Mayer & Heidelberger (1946), and Boursnell, Dewey, Francis & Wormall (1947) those of artificially phosphorylated serum proteins. Kesztyus & Kocsis (1942) have also examined the immunological properties of casein after dephosphorylation with dilute alkali, and compared these properties with those of the same material after rephosphorylation with phosphorus oxychloride (POCl_3), and with those of casein.

In all these cases, however, interpretation of the results is complicated by the unknown factor of the degree of change effected in the protein molecule by the somewhat drastic chemical treatment to which it has been subjected.

The investigations of Francis & Wormall (1948, 1950) on the immunological properties of lipovitellin were directed mainly towards elucidating the respective parts played by the phospholipin and the phosphoprotein of the antigen complex, but the discovery by Mecham & Olcott (1949) of the new phosphoprotein, 'phosvitin,' in hen's egg yolk, has rendered it desirable that these investigations should be extended in order to obtain more information on the nature of the protein part of the complex.

Phosvitin is a protein of relatively low molecular weight, containing about 10% P, and accounting for at least 60% of the protein P of egg yolk. Mecham & Olcott (1949) infer that most, if not all, of the P of vitellin is due to phosvitin.

Experiments have therefore been undertaken on the exchange of radioactivity, *in vitro*, between lipovitellin and a ^{32}P -containing phosvitin preparation, and the immunological and serological properties of phosvitin itself have also been investigated.

MATERIALS AND METHODS

Lipovitellin solutions. These were prepared as described by Francis & Wormall (1948).

Phosvitin solutions. These were prepared as described by Mecham & Olcott (1949). Some of these preparations were made from the yolks of eggs laid by a hen 3-6 days after it had received an intramuscular injection of 2 mc. ^{32}P as Na_2HPO_4 .

Phosphorylated proteins. Phosphorylated proteins were obtained, as described by Boursnell, Dewey & Wormall (1948), by the action of POCl_3 (0.5 g. POCl_3 /g. protein) on human serum or plasma, rabbit and chicken serum, and on the mixed globulins obtained from horse serum.

Alum-precipitated antigens. Some of the antigens were prepared following the method of Proom (1943). Approximately 2% solutions of phosphorylated human serum proteins and of phosvitin were treated with equal volumes of 10% (w/v) potassium alum. The pH was adjusted to 6.5 with 5N-NaOH, and the precipitate centrifuged, washed with 0.9% (w/v) NaCl containing 0.02% merthiolate and finally made up with merthiolated saline to the same volume as the original phosphoprotein solution.

Antisera. Antisera to ethanol-ether extracted, lipid-free vitellin were prepared as described by Francis & Wormall (1948). These antisera react well with lipovitellin, but contain somewhat less of the antibodies reacting principally with the phospholipin part of the lipoprotein complex, than do antisera to lipovitellin. Only aged sera, which had lost these 'antiphospholipin' antibodies (Francis & Wormall, 1950), were used in the experiments reported here. Antisera to phosphorylated human serum proteins were obtained by intramuscular injection of 5 ml. of the alum-precipitated antigen into each hind leg of a rabbit, at intervals of 10-12 days, satisfactory antisera usually being obtained 10 days after the second injection. Sera were also collected from rabbits which had received courses of injection of phosvitin, (a) by intramuscular injection of 10 ml. of alum-precipitated antigen suspension at intervals of 10 days, (b) by intravenous injection of 2 ml. of approx. 2% phosvitin solution, or (c) by intraperitoneal injections of 5 ml. of 2% phosvitin solution, at weekly intervals for 1 month. No differences were observable in the results with the sera obtained by the three methods.

Quantitative precipitin reactions. The antiserum (usually 0.5-1 ml.) was mixed with antigen solution (0.5-3 ml.) and sufficient 0.9% NaCl solution to bring to a standard volume. When phosvitin was used as an inhibitor it was added to the antiserum before the antigen. When lipovitellin was used as antigen, solid NaCl was added (0.1 g./ml.) to the antiserum and to the phosvitin solution, and 10% (w/v) NaCl was

substituted for 0.9% NaCl, to prevent precipitation of lipovitellin and to minimize non-specific precipitation by phosvitin. The mixtures were kept at 37° for 30 min.-1 hr., overnight at 0-4°, and centrifuged at 0°. The precipitates were then washed three times with ice-cold 0.9 or 10% NaCl, dissolved in 0.5 ml. 0.1 N-NaOH, and transferred to micro-Kjeldahl flasks for incineration.

Complement fixation tests and qualitative precipitin tests. These were carried out as described by Berenblum & Wormald (1939) and Hopkins & Wormald (1933) respectively. When lipovitellin was used as antigen for complement fixation tests, the progressive threefold dilutions with 0.9% NaCl were made from a fresh 0.05% suspension in 0.5% NaCl, obtained by diluting with water a 0.25% solution in 2.5% NaCl.

Total N determinations. These were carried out as described by Banks, Francis, Mulligan & Wormald (1951).

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

Non-lipid or 'protein' P determinations. The lipovitellin solution (containing about 0.1 mg. P) was pipetted into 10 vol. 7:3 ethanol-ether mixture. The precipitate was centrifuged, washed twice with 5 ml. ethanol-ether mixture, dried in a current of warm air and incinerated with 1.2 ml. 60% HClO₄.

Radioactivity determinations. These were made by evaporating the radioactive solutions, together with 3 drops of a 10% suspension of kaolin in 50% ethanol, on 14 mm. diameter nickel planchettes. The dried preparations were then counted under a standard 'bell'-type counter with a Panax amplifier and scaler unit.

solution in presence of ³²P-containing phosvitin should contain ³²P. If the percentage of phosvitin originally present in the lipovitellin is unchanged by this treatment, no change in the ratio of protein P to total P should result; whereas any radioactivity in the precipitate due to co-precipitation of unchanged, non-radioactive, lipovitellin and ³²P-containing phosvitin would be accompanied by a corresponding increase in this ratio.

Two 12 ml. portions of an approximately 1% solution of lipovitellin in 10% NaCl were each mixed with 1.2 ml. of an approximately 1% solution of ³²P-containing phosvitin, and immediately poured into 240 ml. water. The precipitated lipovitellin was separated by centrifugation, redissolved in 6 ml. of 20% NaCl solution and again poured into water (240 ml.). The solution and reprecipitation were repeated twice more. After the second and third precipitations, portions of the precipitates (approx. one-third and one-half respectively) were reserved for analysis, and the volumes of 20% (w/v) NaCl and water were reduced in proportion at each subsequent reprecipitation. The twice, thrice and four times precipitated samples of lipovitellin were then dissolved separately in 1 ml. 0.1 N-NaOH, transferred quantitatively to measuring flasks, and made up to 3 ml. Duplicate determinations of radioactivity, total P and non-lipid P were then made on 0.4, 0.4 and 0.6 ml. samples of each solution. A third sample of lipovitellin was treated in the same way, but without the addition of phosvitin, and analysed after four precipitations. This repeated reprecipitation caused no measurable change in the ratio of protein P to total P.

Table 1. *Exchange of radioactivity between lipovitellin and ³²P-containing phosvitin*

(System: Lipovitellin in 10% NaCl solution, 12 ml. (1.588 mg., total P, 0.647 mg. (40.7% of total) protein P). ³²P-containing phosvitin, 1.2 ml. (1.24 mg. P=65.6% of total protein P in mixture). Lipovitellin precipitated from mixture by water (240 ml.), redissolved in 20% NaCl and reprecipitated, four times in all. The letters (a) and (b) refer to separate experiments.)

	No. of precipitations	Protein P (as % of total P)		Lipovitellin protein P replaced by radio-phosvitin P* (%)
		Non-lipid P (total protein P)	Radio phosvitin P	
Lipovitellin	1-4	40.7	0.0	—
Lipovitellin + phosvitin	2	(a) 51.4	37.1	57.1
		(b) 50.0	30.1	42.0
	3	(a) 46.8	26.7	45.0
		(b) 43.8	25.1	51.6
	4	(a) 44.3	24.9	49.2
		(b) 45.4	25.8	47.8

* These figures were calculated after making allowance for the additional phosvitin carried down by the precipitate; had there been complete exchange, all figures in this column would be 65.6.

RESULTS

The exchange of radioactivity between lipovitellin and ³²P-containing phosvitin

If any part of the phosphorus in a lipovitellin preparation is due to the presence of phosvitin, which dissociates from it in solution in 10% NaCl and recombines when the lipovitellin is reprecipitated, then lipovitellin precipitated from 10% NaCl

The results of the two experiments (Table 1) show that there is a very rapid interchange under the conditions of the experiment. The percentage of protein P in the precipitate is somewhat greater than that in the original lipovitellin, particularly after only two precipitations. This is apparently due to some additional phosvitin adhering strongly to the precipitate; but even after allowance has been made for this additional phosvitin P, 48% of the

remaining protein P in the precipitate was derived from the originally free phosvitin, compared with a figure of 65·6 % theoretically possible if there had been complete equilibration between the phosvitin P and vitellin P. In order further to eliminate the possibility of the result being due to adsorption, or combination of phosvitin with the precipitated lipovitellin, the experiment was repeated using equal volumes of 1 % solutions of lipovitellin and radioactive phosvitin, and again the radioactivity of the separated lipovitellin was about 75 % of the maximum theoretically possible.

The action of phosphoprotein antigens on 'antiphosvitin' sera

Qualitative precipitin tests on 'antiphosvitin' sera, using phosvitin as antigen, showed no visible precipitate after 1 hr. at 37°. When either lipovitellin or phosphorylated horse serum globulin were used as antigen, however, a very faint precipitate was discernible in most cases. In quantitative precipitin reactions, 2 ml. portions of several different sera were treated with lipovitellin or phosphorylated globulins to give maximal precipitation as nearly as could be judged. In no case was more than 9 µg. nitrogen precipitated by phosphorylated protein, or 5 µg. nitrogen by lipovitellin, per ml. serum. (In one case, 1 ml. of 'antiphosvitin' serum treated with 0·1 ml. 0·25 % phosvitin gave a precipitate containing 25 µg. nitrogen, but in view of later experiments it is considered that this was probably due to non-specific precipitation.) Control experiments were carried out with normal rabbit serum in place of 'antiphosvitin', and although no visible precipitates were obtained, the tubes were treated exactly as before. The total nitrogen in these 'precipitates' was never greater than 2·5 µg./ml. serum.

No evidence of complement fixation could be obtained when lipovitellin or phosvitin were mixed with 'antiphosvitin' sera in presence of guinea pig serum, although the lipovitellin gave strong precipitin and complement fixation reactions with antivittellin serum.

The action of phosvitin on antisera to phosphoproteins

Qualitative precipitin tests were carried out with phosvitin as test antigen, using antisera to phosphorylated proteins and to vitellin. Complement fixation tests were also carried out with the phosvitin-antivitellin system, but in no case was any reaction discernible. (All the phosvitin preparations tested were found to be slightly 'anti-complementary', possibly due to combination or co-precipitation occurring between phosvitin and a protein component of complement.)

The effect of phosvitin on phosphoprotein precipitin systems

Although phosvitin does not precipitate antibodies to phosphorylated proteins or to vitellin, this does not preclude the possibility that it might react with them. Experiments were therefore carried out to determine whether phosvitin had any inhibitory effect on precipitin reactions between these antibodies and appropriate antigens.

Table 2. *Effect of phosvitin on the phosphorylated protein precipitin system*

(System: 1 ml. rabbit antiserum to phosphorylated human serum proteins; 0·5 ml. phosphorylated horse globulin solution (200 µg. N), 0·9 % NaCl added to each tube to give a total volume of 3·5 ml. after addition of phosvitin.)

Phosvitin added (µg. N)	Total N in precipitate (µg.)			
	Complete system	Control with no antiserum	Control with no antigen	Control with normal serum
0	62	—	—	—
144	58	4	5	5
288	62	5	6	7
576	97	4	17	20

Fig. 1. The effect of addition of phosvitin to lipovitellin-antivitellin-precipitin systems. The dotted lines denote the addition of phosvitin, continuous lines the addition of lipovitellin, to 1 ml. of antivittellin serum. In control experiments corresponding to the points marked with an arrow, using normal rabbit serum instead of antivittellin serum, large and variable amounts (0·18–0·4 mg. N) were obtained, due to non-specific precipitation by phosvitin.

(a) *Phosphorylated proteins systems.* The effect of phosvitin on the precipitation of an antiserum to phosphorylated human serum proteins by phosphorylated horse serum globulin, phosphorylated rabbit serum proteins and phosphorylated human

plasma proteins, was investigated qualitatively. In these experiments quantities of phosvitin up to four times the weight of antigen used were added to the precipitin systems, but in no case could inhibition be detected by visual inspection. In some cases, however, there appeared to be an increase in the amount of precipitate when the larger amounts of phosvitin were used. A quantitative experiment was therefore carried out, the results of which are shown in Table 2. These figures show no evidence of inhibition by phosvitin. The increased precipitation when the largest amount of phosvitin was used is found also in the control systems, and is therefore almost certainly due to non-specific co-precipitation between phosvitin and normal serum proteins.

(b) *The lipovitellin-antivitellin system.* Quantitative precipitin reactions were carried out in which (i) varying amounts of lipovitellin, and (ii) varying amounts of phosvitin together with a fixed amount of lipovitellin, were added to a fixed volume of an aged antiserum to vitellin. The results are shown in Fig. 1. Again there is no evidence of inhibition, but an increase in the amount of precipitate due to non-specific precipitation was obtained when large amounts of phosvitin were added.

DISCUSSION

The artificial phosphorylation of proteins by treatment with phosphorus oxychloride (Bechold, 1901; Neuberg & Pollak, 1910; Neuberg & Oertel, 1914; Rimington, 1927) is always accompanied by some denaturation, and probably causes significant changes in the protein molecule besides the introduction of phosphate groups. The changes in the immunological properties of ovalbumin caused by this treatment (Heidelberger *et al.* 1941) may therefore be due to a variety of causes. Phosphorylation of serum proteins (Mayer & Heidelberger, 1946; Bournsnel *et al.* 1947, 1948) results in products whose phosphate groups are fairly rapidly lost under physiological conditions of temperature and pH, but these products, when injected into rabbits, in some of our experiments, produced antisera still capable of reacting with other artificially phosphorylated proteins. In the present series of investigations, in an attempt to retain the intact phosphorylated serum proteins in the tissues for a longer time after injection, the technique of intramuscular injection of alum-precipitated antigens (Proom, 1943) has been used. The injection of alum-precipitated phosphorylated human serum proteins has resulted in the production of antisera reacting weakly with phosphorylated rabbit serum, chicken serum and horse serum globulins, but these antisera do not react, either in precipitin or complement fixation reactions, with phosvitin or lipovitellin. Furthermore, the addition of phosvitin to a phos-

phorylated protein precipitin system does not result in any diminution in the amount of precipitate obtained, although it might be expected that phosvitin, even though it does not precipitate antibodies to phosphoprotein, would be capable of inhibiting formation of a specific precipitate in any system where the primary reaction was due to the presence of phosphate groups in the antigen.

These results therefore indicate that very little, if any, of the immunological properties of artificially phosphorylated proteins are likely to be due directly to the introduction of phosphate groups into the molecule.

Francis & Wormall (1948, 1950), have shown that the lipoprotein complex, lipovitellin, on injection into rabbits, produces antisera capable of specific precipitation with lipovitellin; the specificity of the antibodies produced is, however, directed more towards the phospholipin part of the complex than the phosphoprotein part. These antisera, on ageing, lost much of their antibodies reacting primarily with lipids, and thereafter precipitated phospholipin and phosphoprotein in approximately the same ratio as that in the original antigen. Owing to the insolubility of vitellin, however, it was impossible to use the lipid-free antigen in quantitative precipitating reactions, and therefore it could not be ascertained what parts the phosphoprotein and phospholipin played respectively in the precipitin reaction with these aged antisera.

If the inference of Mecham & Olcott (1949) regarding the identity of the phosphorus of vitellin and phosvitin is correct, this difficulty should be overcome by using phosvitin in place of vitellin. However, the use of phosvitin as a test antigen is complicated by the fact, observed by these authors, that it possesses the property of precipitating non-specifically with other proteins, e.g. protamine and bovine serum albumin. These precipitates are soluble in 2N-sodium chloride, like lipovitellin, but precipitate at lower salt concentrations.

The increase in the amount of precipitate in a lipovitellin-antivitellin system, on adding relatively large amounts of phosvitin, is almost certainly due to this non-specific precipitation, and the failure to cause either increase or inhibition at lower phosvitin concentrations indicates that phosvitin plays little or no part in the immunological reactions of lipovitellin.

This is further confirmed by the finding that phosvitin and antivitellin serum incubated with guinea pig serum showed no fixation of complement, although the same antiserum reacted strongly, in both precipitin and complement fixation tests with lipovitellin.

The conditions used by Mecham & Olcott (1949) in their isolation of phosvitin, involving nothing

more drastic than treatment with 0.4 M-magnesium sulphate and saturated ammonium sulphate, are not such as would lead one to expect degradation of the natural phosphoproteins of the egg yolk. Their inference that the protein P of samples of vitellin is largely, if not entirely, due to the presence of small amounts of phosvitin is, on these grounds, not unreasonable. On the other hand, Osborne & Campbell (1900) have reported the isolation from peptic digests of vitellin of a product, in 25 % yield, containing all the vitellin P, and in addition, 1 % of sulphur. Since phosvitin is resistant to peptic digestion and contains no sulphur, it could not have been the mother substance of this product, and it appears unlikely that this product, which contained 3-4 % P, could have been phosvitin contaminated with twice its weight of P-free protein. Other workers (Levene & Alsberg, 1900; Swigel & Posternak, 1927*a*) have reported the isolation of vitellin degradation products of high P-content similar to phosvitin, and Swigel & Posternak (1927*b*) have also reported the presence in egg yolk of an enzyme capable of liberating polypeptides of high P-content from vitellin. On these grounds, therefore, the possibility of phosvitin being a degradation product of vitellin cannot be entirely excluded. Neither vitellin nor phosvitin are believed to be homogeneous proteins, and the composition of different lipovitellin preparations certainly varies slightly. However, a lipovitellin preparation which has been several times reprecipitated is unlikely to contain an enzyme which could produce phosvitin from it sufficiently rapidly to account for the exchange reaction reported here, and at the same time leave most of the original material still in a readily precipitable form. It therefore seems highly improbable that such a preparation would undergo exchange of P with a phosvitin preparation unless the lipovitellin did actually contain phosvitin. In the experiments reported here, this exchange did occur, to the extent of about 75 % of that theoretically possible if all the vitellin P were due to phosvitin, and the same result was obtained whether the ratio of phosvitin P to vitellin P in the mixture was 2:1 or 20:1, and whether the lipovitellin was precipitated immediately after mixing, or after incubating at 37° for 1 hr.

The lipovitellin precipitated from a lipovitellin-phosvitin mixture contained more protein P, as a percentage of the total P (45 % as against 41 %) than did the original lipovitellin, indicating that some phosvitin had combined fairly firmly with the vitellin part of the lipoprotein complex. This additional protein P is, however, incapable of accounting for more than a fraction of the radioactivity in the precipitate, most of which must therefore have arisen from an exchange of P between the two components of the system. It is

inconceivable that the phosphate groups of the two proteins should have exchanged under the experimental conditions employed, and the result therefore confirms Mecham & Olcott's inference, i.e. about 75 % of the protein P of vitellin appears to be due to phosvitin.

No antisera have yet been found to give specific precipitates with phosvitin, neither does phosvitin act as a specific inhibitor with lipovitellin or phosphorylated protein precipitin systems. It is therefore hardly surprising that it is incapable of producing antisera when injected into rabbits. This lack of antigenicity might be due to its relatively simple structure, and like gelatin, the molecule may lack the rigidity which appears to be necessary for antigenicity. On the other hand, it is quite possible that, since a large proportion of the molecule (about 50 %) is composed of phosphoserine groups, the structure of the remainder of the molecule does not differ sufficiently from that of phosphoproteins of similar function which presumably also occur in other species of animal. In this connexion it should be noted that Harris (1946) has reported the occurrence of a phosphoprotein in frog spawn, and Roepke & Bushnell (1936) have detected by immunological methods traces of a protein, similar to vitellin, in the serum of cocks, and more in that of laying hens. In the experiments reported here, twelve rabbits were used for the preparation of 'antisera' to phosvitin, six of either sex, and no differences were observed between the behaviour of the males and females.

The apparent traces of a precipitin reaction obtained in a few cases between 'antiphosvitin' sera and lipovitellin or phosphorylated proteins, which could not be confirmed in complement fixation tests, were probably not significant, and might have been due to traces of some impurity, such as a denatured protein, giving reactions with some non-specific antibody present in the serum.

The antigenicity of vitellin would therefore appear to be due to the non-phosvitin component of the protein.

SUMMARY

1. Phosvitin containing ^{32}P has been prepared, and shown to undergo an exchange reaction with lipovitellin in 10 % sodium chloride solution, to the extent of about 75 % of that theoretically possible if all the phosphorus of the vitellin were due to phosvitin.

2. Phosvitin has been shown not to react, either in precipitin or complement fixation reactions, with antisera to artificially phosphorylated proteins or to vitellin.

3. Phosvitin does not inhibit precipitate formation in lipovitellin-antivitellin or phosphorylated protein precipitin systems.

4. Injection of phosvitin, either intravenously or intraperitoneally, or intramuscularly in the form of an alum precipitate, into male or female rabbits does not produce sera capable of reacting in precipitin or complement fixation reactions with phosvitin, lipovitellin, or artificially phosphorylated proteins.

5. The significance of these results is discussed, in

relation to the composition of vitellin and the structure of phosvitin.

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