The Use of Radioactive Isotopes in Immunological Investigations

4. ANTIBODIES TO PHOSPHOLIPINS

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In an earlier study we found that precipitates obtained from mixtures of lipovitellin and antisera to lipovitellin contained relatively more phospholipin than did the lipovitellin used as antigen (Francis & Wormall, 1948). This combination (or adsorption) of phospholipin by the antigen-antibody complex was shown to be specific, indicating the presence in the antiserum of antibodies reacting with phospholipin.

The injection of lecithin does not invariably lead to antibody formation, even when it is mixed with the serum of a foreign species (cf. reviews by Marrack, 1938; Landsteiner, 1945, p. 111; Boyd, 1947), but the antigenicity of many complexes containing protein (or polypeptide), carbohydrate and lipid (e.g. the 'Boivin antigens') is well established. Little information is available, however, about the serological properties of the phospholipin fraction of lipoproteins, and we decided, therefore, to make a further study of our anti-lipovitellin sera from this viewpoint.

In further tests with samples of the anti-lipovitellin sera which we had prepared previously (Francis & Wormall, 1948) and stored in a sterile condition, we found that these antisera had lost all, or practically all, their capacity to carry down extra phospholipin in the antigen-antibody precipitates, i.e. they appeared to have lost their antibodies for phospholipins. Tests were therefore made with many samples of these antisera, some preserved in the filtered liquid state and some in the freeze-dried state, and the results of these experiments are described below.

EXPERIMENTAL

Determinations of ^{32}P were made as described (Francis & Wormall, 1948), but the counter described by Veall (1948) was used for the determination of the radioactivity of solutions (e.g. ethanol-ether solutions of phospholipins, supernatant solutions from precipitin tests, and occasionally solutions of lipovitellin or vitellin in very dilute NaOH). Ordinary P determinations were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Lipovitellin. ^{32}P -containing lipovitellin was made from the yolks of eggs laid by a hen 4–10 days after it had received an intramuscular injection of 500 μc . of ^{32}P as a neutralized solution of sodium phosphate (Francis & Wormall, 1948). The four preparations (A, B, C and D) used in these experiments gave 2500, 5000, 1300 and 5000 counts/min./mg. P with our Geiger-Müller counter system.

Lipid P and vitellin P of lipovitellin. In the preparation of lipovitellin ('soluble' vitellin; Francis & Wormall, 1948) the free lipid of egg yolk is removed by extraction with ether (containing 2% ethanol), but there still remains much phospholipin which can be removed by more drastic extraction. Nearly half the P of lipovitellin can, for example, be removed by extraction with hot ethanol (Calvery & White, 1932) or by treating the solution of lipovitellin, in 5–10% NaCl, with 5 vol. of an ethanol-ether mixture (7:3, v/v) and washing the precipitated vitellin with the solvent mixture and then with water (Francis & Wormall, 1948). For the purposes of this paper the P extracted from lipovitellin by the ethanol-ether mixture is called lipid P, and that not extracted, the vitellin P. Our results show that extraction with cold ethanol-ether removes all the lipid P, since no more can be extracted by a subsequent extraction with hot ethanol (Table 1).

The distribution of P in lipovitellin preparations A, B and C, and in precipitates obtained with them, was determined by radioactivity determinations. This is justifiable, since it is most unlikely that 'physical exchange' of radioactive phosphate will occur between phospholipins and phosphoproteins, for such an exchange does not readily occur (cf. review by Hevesy, 1948). The results were calculated by comparing the radioactivity of the fractions with that of the original lipovitellin, and the corresponding values (Tables 1 and 2) have been calculated on the assumption that the $^{32}\text{P}/^{31}\text{P}$ ratio for the phospholipins was the same as that for the vitellin. With the lipovitellin used in this work the two ratios were not widely different, and furthermore we were mainly interested in a comparison between the lipid P/vitellin P ratio for a lipovitellin-anti-lipovitellin precipitate and that for the lipovitellin used as antigen in that particular precipitin test. It should not be overlooked, however, that the specific activity of the P of the yolk phospholipins of eggs laid by hens injected with ^{32}P is not necessarily the same as that of the P of the protein component of the lipovitellin. Chargaff (1942), for example, found that the vitellin P had a considerably higher specific activity than had the lipid P in the eggs laid during the first 5 or 6 days after the injection of ^{32}P . With our lipovitellin preparation D, the specific activity of the vitellin P was 1.17 times that of the lipid P, and if allowance is made for a difference in this direction the only effect is to increase slightly the difference between the lipid P/vitellin P ratio for the precipitate and that for the antigen in the experiments recorded in Table 2.

Antisera. These were prepared by intraperitoneal injection of lipovitellin solutions into rabbits (Francis & Wormall, 1948). Samples of the fresh untreated sera were used for some of the precipitin tests, and other precipitin tests were made with samples preserved as follows: (a) filtered through a Berkefeld filter and kept in sterile tubes at 3–5°, and

(b) freeze-dried in the apparatus described by Greaves (1946) and kept in sealed tubes at room temperature. The preserved freeze-dried sera dissolved in water to give clear solutions, but heavy precipitates gradually separated from the filtered sera (a) on keeping. All cloudy sera were centrifuged, and the clear supernatant solutions used for the precipitin tests.

Precipitin tests. These were carried out as described (Francis & Wormall, 1948), using the proportions of antigen and antibody required to give approximately optimal precipitation. Radioactivity determinations were made on the precipitates (washed with 10% (w/v) NaCl and then dissolved in a few drops of 0.5N-NaOH) and the supernatant solutions. Some of the precipitates were extracted well with ethanol-ether, and ^{32}P -determinations were made on the extracted precipitate (to give the vitellin P) and on the extract (lipid P).

RESULTS

The phospholipin content of lipovitellin

Lipovitellin preparations vary appreciably in their phospholipin content, and this variation might be due partly to the severity and the duration of the extraction of the egg yolk with ether, and partly to

Table 1. *Lipid P content of lipovitellin solutions*

(The solution of lipovitellin in 10% (w/v) NaCl was treated with 5 vol. of 7:3 (v/v) ethanol-ether. The precipitate was washed twice with ethanol-ether and once with water, and dissolved in a few drops of 0.5N-NaOH for ^{32}P determinations. In some of the experiments with preparation C, samples of the stock solution were diluted with 31 vol. of 10% NaCl before treatment with ethanol-ether.)

Preparation	Total* P ($\mu\text{g./ml.}$)	Vitellin† P ($\mu\text{g./ml.}$)	Lipid† P as % of total P
A	157	82.5	48
B	50.8	30.1	41
C	1315	455	65
	1315	418	68
	41.1	12.8	69
	41.1	14.8	64
	1315	435†	67
	1315	489†	63
	41.1	15.3†	63
D	251	96*	62*

* Results obtained by colorimetric P determinations; all the other P contents recorded in this table are calculated from ^{32}P determinations (see the text, p. 380).

† The term lipid P is used for the P which can be extracted by an ethanol-ether mixture. The vitellin P is that which is not extracted by this solvent mixture.

‡ These preparations were further extracted by boiling for 1 hr. with 10 vol. of absolute ethanol before the ^{32}P content was determined.

differences between individual eggs. The free, but not the combined, lipid in the yolk is fairly readily extracted by ether. The combined lipid has to be 'released' in some way from the lipoprotein complex, and this can be effected by hot ethanol (Calvery & White, 1932), or, as we have found, by extraction with an ethanol-ether mixture at room temperature or even at 0°.

The lipid P of most of the lipovitellin preparations analysed accounts for one-half to two-thirds of the total P, and the data for four preparations used in the immunological experiments described here are given in Table 1. Preparation B was a little different from the others, for only 41 % of its total P was present as lipid P, but preparations A, C and D, and others not described here, gave average values between 48 and 66 %.

Tests made with preparation C showed that treatment and extraction with cold ethanol-ether is sufficient to remove all extractable phospholipin. Averaging the results of four determinations, it was found that 66 % of the total P of the lipovitellin was extracted by the ethanol-ether mixture, and the corresponding value for the samples of the same material extracted with ethanol-ether followed by hot ethanol was 64 %. Other experiments with preparation C showed that the percentage of P removed by ethanol-ether does not depend on the concentration of the lipovitellin in the aqueous solution; e.g. the percentage of lipovitellin P extracted by ethanol-ether was the same for the undiluted lipovitellin solution as it was for the same solution diluted with 31 vol. of 10 % NaCl (Table 1).

The phospholipin content of lipovitellin- anti-lipovitellin precipitates

The results of a series of precipitin tests with rabbit antisera to lipovitellin are given in Table 2. It will be seen that, although the fresh antiserum gave precipitates which contained considerably more lipid P, as a fraction of the total P, than did the lipovitellin used as antigen, this difference was not observed when a sample of the same antiserum was tested at a later date. The sterile filtered antisera had all developed heavy precipitates during storage in the refrigerator, and it was concluded that the antilipid antibodies had been removed in the precipitate. Further tests with samples of the same and similar antisera, kept under various conditions, fully confirmed this view. Antisera which had been kept in the freeze-dried condition behaved exactly like fresh anti-lipovitellin sera; they gave precipitates in which lipids accounted for 83 % of the total P, as against a corresponding value of 66 % for the lipovitellin used as antigen.

These differences between old and fresh 'liquid' anti-lipovitellin sera are even more striking when the P distribution is expressed as a lipid P/vitellin P ratio. The ratios for the precipitates with fresh and freeze-dried antisera were 2.6 and 4.5, respectively, compared with corresponding ratios of 0.9 and 1.9 for the appropriate lipovitellin antigens. With the old sera kept in the liquid state the average ratio for the precipitates (1.7) was actually a little less than that for the corresponding lipovitellin (1.9).

Table 2. *Phospholipin content of lipovitellin-anti-lipovitellin precipitates*(All the weights in this table are given as $\mu\text{g./ml.}$ of antiserum used.)

Anti-lipovitellin serum		Antigen (lipovitellin) added		Composition of precipitate		
		Total P ($\mu\text{g.}$)	Lipid* P as % of total P (%)	Lipid* P ($\mu\text{g.}$)	Vitellin* P ($\mu\text{g.}$)	Lipid* P as % of total P (%)
No.	Treatment					
457	Fresh and unfiltered†	787.5	48	134.5	54	71
		523.5	48	123.5	46.5	73
	Fresh and unfiltered†	762	41	29	8	78
		508	41	45	11.5	80
		381	41	39.5	11.5	78
	Old, filtered‡	205.5	66	22	10	69
				21.5	11.5	65
		100	66	8.0	4.7	63
				7.4	5.1	59
	Old (filtered‡), warmed at 37°	175	66	13	7.3	64
				12.3	9.0	58
				12.7	7.0	64
	541	Old, freeze-dried	175	66	45.7	10
				45.7	10	82
				49.3	9.7	84
Antiserum to iodinated globulin						
No.	Treatment					
602	None	205.5	66	0	0	0
				0	0	0

* Determined by radioactivity measurement (see the text, p. 380). See also the second footnote to Table 1.

† Data from Francis & Wormall (1948).

‡ Filtered through a Berkefeld filter.

Control tests with the lipovitellin solution used in most of these precipitin reactions showed that no detectable ^{32}P -containing material was precipitated when the lipovitellin solution was mixed with a rabbit antiserum to iodinated horse-serum globulin (Table 2); and in a previous investigation (Francis & Wormall, 1948) it was shown that in a system containing a considerable amount of lipovitellin, no significant amount of lipovitellin phospholipin was carried down by a heavy serological precipitate unrelated to lipovitellin. Thus the precipitation of extra phospholipin by the anti-lipovitellin serum is specific.

In another investigation a study was made of the composition of lipovitellin-anti-lipovitellin precipitates obtained with the antigen labelled with ^{32}P and the antibodies with ^{131}I . Although this experiment was designed for a different purpose, some of the results are pertinent to this study of anti-lipids, and are given in Table 3. They are of special interest in that the separated globulin fraction of the antiserum was used, whereas all the other precipitin tests described here were made with the whole antiserum. It is true that the antibodies were modified slightly in that they were labelled with ^{131}I , but the amount of iodine introduced was insufficient

to effect any detectable change in the properties of the antibodies; certainly the iodination cannot have produced antibodies specific for phospholipins.

The results of these experiments with the globulin fraction of the antisera showed that lipids accounted for 82% of the total P of the lipovitellin-anti-lipovitellin precipitates, compared with 62% for lipovitellin. The lipid P/vitellin P ratios were 4.3–5.2 for the precipitates, and 1.6 for the lipovitellin.

DISCUSSION

The combination between phospholipin and vitellin in the lipovitellin complex is relatively firm, but it can undoubtedly be broken by only moderately drastic treatment of the complex, e.g. with ethanol or ethanol-ether mixtures (but not with ether alone). It is not inconceivable that the union of antibody and antigen might involve energy changes sufficient to effect this disruption of lipovitellin, but it would be unwise to discuss this possibility until more is known about the chemistry of the egg-yolk phosphoproteins (cf. for example, the recent work of Mecham & Olcott 1949).

The hypothesis that fresh antisera to lipovitellin contain antibodies which have their reactive groups

directed primarily towards the phospholipin, and not to the lipoprotein complex as a whole, is supported by our present evidence. However, these antisera to lipovitellin lose much of these anti-lipid antibodies when they are kept in the liquid state in the cold, and this loss may well be due to gradual precipitation of the anti-lipids by the ordinary lipids in the antiserum.

Table 3. *Composition of some precipitates obtained from mixtures of lipovitellin and the globulin fraction of rabbit antisera to lipovitellin*

(A solution (1 vol.) of a freeze-dried anti-lipovitellin serum (mixed sera) was mixed with 1 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution and the precipitated globulins were dialysed against 0.9% NaCl at about 4° until free from $(\text{NH}_4)_2\text{SO}_4$. After the addition of 0.9% NaCl to give 0.5 vol. the antibodies were labelled with ^{131}I by very mild iodination (final vol. of 1 vol.). Differential radioactivity determinations measured the amount of antigen and antibody in the various precipitates, but the values for antibody are omitted from the table below since they have no bearing on the problems discussed in this paper. All the weights given in this table are in $\mu\text{g./ml.}$ of antiserum used.)

Antigen (lipovitellin)		Composition of the lipovitellin- anti-lipovitellin precipitate		
Total* P ($\mu\text{g.}$)	Lipid* P as % of total P (%)	Lipid P ($\mu\text{g.}$)	Vitellin P ($\mu\text{g.}$)	Lipid P as % of total P (%)
125	62	26.6	5.8	82
167	62	30	6.9	82
209	62	37.2	7.6	83
251	62	40	7.6	84

* Results obtained by colorimetric P determinations; all the other figures in this table are calculated from measurements of radioactivity compared with those of standard preparations of lipovitellin and of vitellin, and are therefore independent of differences in the $^{32}\text{P}/^{31}\text{P}$ ratios of vitellin and phospholipin.

Our antisera to lipovitellin contained very little phospholipin (less than one-tenth that in our other antisera and normal rabbit sera), and the amount in the precipitate which separated from 6 ml. of antiserum on storage was undetectable. Significant amounts of lipid were, however, present in this precipitate and it is not improbable that our antibodies to phospholipin are also capable of reacting with other lipids. Precipitate formation in these antisera may perhaps be merely a particular aspect of the functioning of the globulin antibody as a 'transporteur' of lipid in the sense suggested by Grabar (1947); thus a higher ratio of 'transporteur' to lipid than that found normally in serum might well bring the mixture into the zone where precipitation occurs, in a manner analogous to that observed with varying antigen-antibody ratios in other systems.

Lipids undoubtedly take part in many, but not all, serological precipitations. Thus Hartley (1925) showed that normal human or horse serum proteins are not precipitated by their antisera when both antigen and antiserum are freed from ether-soluble constituents. Horse serum pseudo-globulin antigen-antibody precipitates, on the other hand, contain no detectable lipid (Marrack & Smith, 1931), and several other specific precipitates contain little lipid (see review by Landsteiner, 1945). In our experiments the possibility of non-specific carrying down of phospholipin by the antigen-antibody complex can virtually be excluded, since the phospholipin of lipovitellin is not carried down by unrelated antigen-antibody precipitates.

It is not suggested that our results support the view that the lecithins and other phospholipins are fully antigenic. Since there is apparently little, if any, difference between the phospholipins of hen eggs and those of the mammalian body, it would be strange if the injection of egg phospholipins into a rabbit were to produce specific antibodies. Phospholipin firmly attached to vitellin would, however, be in quite a different category, and this 'foreign' complex might readily engender several different types of antibody, some reacting with the protein and some with the phospholipin. In many respects the antibody response might be analogous to that following the injection of iodinated proteins, when antibodies are formed capable of combining specifically with 3:5-diiodotyrosine (Wormall, 1930), although this compound is a normal constituent of the body. Antibodies specific for the rest of the iodinated protein, if this protein is foreign to the injected animal, are also formed, unless iodination is so complete that the diiodotyrosine groups completely dominate the molecule from the immunological viewpoint.

It would seem possible, therefore, that if lipid-free vitellin, or any other 'foreign' protein which is capable of combining firmly with phospholipin, is injected into the animal body, it might combine with the blood or tissue phospholipins to form a lipoprotein complex foreign to the body. The complex should then stimulate the production of antibodies reacting with phospholipins. Preliminary tests we have made with antisera to vitellin are in full agreement with this hypothesis, but a much more exhaustive investigation will be required before it is established that vitellin completely free from phospholipin can produce, when injected into a rabbit, antibodies capable of reacting with phospholipin.

SUMMARY

1. Lipovitellin containing ^{32}P has been used to study the lipid and vitellin P contents of lipovitellin-anti-lipovitellin precipitates.

2. Fresh anti-lipovitellin rabbit serum gives precipitates which have significantly higher lipid P/vitellin P ratios than has the lipovitellin used as antigen.

3. This ability of the antiserum specifically to react with phospholipins is lost when the sterile serum is kept for some weeks in the refrigerator, probably because of interaction, with precipitate formation, between the antibodies and the normal lipids in the antiserum. These antisera do not lose

their 'antilipid' antibodies when kept in the freeze-dried state.

4. The significance of antibodies to phospholipins is briefly discussed.

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