The Use of Radioactive Isotopes in Immunological Investigations

1. PRECIPITIN REACTIONS WITH OVOVITELLIN CONTAINING P^{32}

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Analysis of the specific precipitates obtained from antigen-antibody mixtures can help to throw light on many problems of immunology; for example, the quantitative relationship between antigen and antibody in the precipitin reaction, the behaviour of 'inhibitory' substances, and the relative avidities of closely related antigens. Total N determinations on the antigen and on the specific precipitates have been used to determine the antibody/antigen ratio (Heidelberger & Kendall, 1929; cf. Heidelberger, 1939), and this method is of particular value when polysaccharide antigens containing little or no N are used. Use has also been made of antigens containing readily determinable elements or groups which are absent from the antibody. Amongst such 'labelled' antigens are haemoglobin (Wu, Cheng & Li, 1929), iodoalbumin (Wu, Sah & Li, 1929), haemocyanin (Hooker & Boyd, 1936) and proteins conjugated with dyes (Heidelberger & Kendall, 1930, 1935). For a somewhat different purpose N^{15} has been used as a tracer for investigations on the formation and the circulation of antibodies (Schoenheimer, Heidelberger, Rittenberg & Ratner, 1941; Schoenheimer, Ratner, Rittenberg & Heidelberger, 1942; Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942).

In the present investigation an attempt has been made to use antigens containing radioactive tracer elements for serological investigations. These elements can be determined in extremely minute amounts, and the determinations are not affected by the presence of relatively large amounts of the corresponding non-radioactive element. Two convenient isotopes for this type of work are S^{35} and P^{32} .

For the preparation of radioactive antigens the following methods were considered: (i) conjugation of a natural protein with a simple organic substance containing the radioactive element; (ii) direct phosphorylation of natural proteins or of dephosphorized casein, using phosphoryl chloride prepared from radioactive P; (iii) a biological preparation of a protein containing the particular element whose radioisotope is to be used (e.g. vitellin containing P^{32} , haemoglobin containing Fe^{55} or Fe^{59}). With regard to the first method, Berenblum & Wormald (1939), and Bournsnel, Francis & Wormald (1946) have investigated the serology of proteins treated with $\beta\beta'$ -

dichlorodiethylsulphone, and Banks, Bournsnel, Francis, Hopwood & Wormald (1946) have described the preparation of mustard gas sulphone-protein derivatives containing radioactive sulphur (S^{35}). We have recently used this S^{35} -containing conjugated antigen, and phosphorylated proteins containing P^{32} , for some preliminary serological investigations, and a brief account of the results has been given elsewhere (Bournsnel, Dewey, Francis & Wormald, 1947). The present paper deals with the preparation of hen ovovitellin containing P^{32} and its use in quantitative precipitin reactions.

METHODS

Collection of radioactive eggs. In the first experiment, two hens (nos. 815 and 816) were each given an intramuscular injection in the right thigh of 50 microcuries (μC) of P^{32} (in the form of neutral sodium phosphate, in 1-1.5 ml. of 0.9% (w/v) NaCl solution). In the second experiment, hen 816 was given a single injection of 380 μC , about 1 month after the first. The eggs were collected for 1 month after the first injection and worked up in small batches (1 or 2 eggs laid on the same day, for the first 12 days, and batches of 7 eggs thereafter). In the second experiment a vitellin preparation of relatively high radioactivity was obtained from the eggs laid on the 4th, 5th and 6th days following the injection.

Separation of vitellin. This was carried out essentially by the method of Calvery & White (1932). The separated and washed yolks were punctured, and the membrane contents allowed to flow out into 20 ml. of 10% (w/v) NaCl per yolk. As much lipid as possible was removed by extraction with ether containing 2% ethanol (6-8 extractions), and the vitellin in the saline solution was then precipitated twice with 20 vol. of tap water. (It was found that some samples of ether caused denaturation of the vitellin, rendering it insoluble in 10% NaCl, but freshly opened bottles of ether, sp.gr. 0.720 (British Drug Houses Ltd.), were satisfactory.) For most purposes the final product was dissolved in 10% NaCl to give a total vol. of 50 ml. per egg, and used in this form ('soluble' or lipovitellin).

In a few experiments, however, further removal of lipid was effected, either by the hot ethanol treatment of Calvery & White (1932) or by pouring the cooled NaCl solution into 5 vol. of a cooled 7:3 ethanol-ether mixture, centrifuging, washing twice with ethanol-ether and twice with water, all these operations being carried out at 0° to -5°. Both extraction processes gave products ('insoluble' vitellin) with approximately the same P/N ratio (about one half that of the lipovitellin), but both rendered the products

insoluble in 10% NaCl. The product obtained by the cold ethanol-ether method was, however, soluble in saturated urea solution.

Preparation of radio-lecithin. The total ether extracts of the radioactive yolks were dried over anhydrous Na_2SO_4 , evaporated to dryness, and the residues redissolved in dry ether. The radio-lecithin was separated from this solution by pouring into acetone and purified by redissolving in chloroform and reprecipitating with acetone. The final product was dried *in vacuo* over CaCl_2 . In some serological tests radio-lecithin was added to an antiserum; this was effected by emulsifying the lecithin with normal rabbit serum, centrifuging and adding the supernatant fluid to the antiserum.

Radioactivity of the eggs. The main object of these experiments was the preparation of radio-vitellin, and at a later stage radio-lecithin, for immunological experiments, but a few measurements on the individual eggs, and on certain fractions of each egg, were made in order to obtain some idea of the rate of elimination of the injected P^{32} in various fractions of the egg. Hevesy & Hahn (1938) and Aten (1939) have studied the radioactivity of the lecithin fraction of the eggs of hens injected with P^{32} , but no specific information about the vitellin was available to us when our investigations were started. Radio-determinations were made on (a) the whole intact egg, (b) the whole yolk, (c) the separated total lipid (i.e. the ethanol-ether soluble fraction of the yolk) and (d) the separated lipovitellin. The values recorded for (a) and (b) do not, of course, represent the total radioactivity of the material concerned, but they serve as a rough comparison of the P^{32} content of the shells and yolks of different eggs and they give some idea of the rate of elimination of the injected P^{32} in these two fractions of the egg.

For the intact egg 'count', the egg was placed directly under the window of the Geiger-Müller counter. After the egg had been broken, the empty shell was, in some cases, roughly reconstituted into its original shape, and was found to have almost the same radioactivity as the original egg; calculations showed that the white of the egg had absorbed over 75% of the radiations from the yolk. The radioactivity of the white was usually so small that it contributed little to the activity of the intact egg. Thus the 'counts' for the intact egg are of value in giving an approximate relative value for the radioactivity of the shell.

The whole washed yolk (and in some cases the whole white) from each egg was placed separately in small glass crystallizing dishes and 'counted'. These values also are merely relative, and they should not be compared with the values for the intact egg or the more accurate figures for the separated lipids and lipovitellin; they merely serve to give an idea of the day-to-day variation in the radioactivity.

For the yolk lipid 'counts', samples of the total ethanol-ether extract from each batch, corresponding to 1/10th of an egg, were evaporated in 20 ml. porcelain crucibles which were then placed under the counter. Similar determinations were made on fractions (each equivalent to 1/50th of an egg) of the final NaCl solution of lipovitellin from each batch, the solution being evaporated on a brass sample plate, and radioactivity determinations made. Tests showed that the amounts of material present in the samples were not sufficient to lead to any significant loss of the P^{32} radiation by 'self-absorption', and since the dimensions, and geometrical arrangements relative to the counter windows, of the lipid and vitellin layers were approximately

the same, a general comparison of the two sets of results seems justifiable.

Immunization. Rabbits were injected with non-radioactive vitellin, two rabbits being immunized with the 'soluble' and three with the 'insoluble'. In each case intraperitoneal injections of 5 ml. of a solution (or suspension) in 10% NaCl were given, containing about 3% of protein and 0.25% phenol (as a preservative), at intervals of 7-10 days. Precipitin tests were carried out on sera taken from the rabbits 7-10 days after the fourth and subsequent injections. Good antisera were obtained from both rabbits injected with 'soluble' vitellin and one injected with 'insoluble' vitellin; the other rabbits yielded weak antisera.

Precipitin tests. Antiserum diluted with an equal vol. of 20% NaCl (0.4 ml.) was mixed with 2.0 ml. of diluted antigen in a 15 ml. tapered centrifuge tube. (The antigen was previously centrifuged for 20 min., and dilutions were made with 10% NaCl to avoid precipitation of the vitellin by a reduction in the salt content.) After 3 hr. at 37°, with occasional stirring, the tubes were centrifuged for 20 min. and the supernatant fluids discarded. The inside of each tube was washed down with 6 drops of 10% NaCl, care being taken to disturb the precipitate as little as possible, and the tubes were centrifuged for a further 7 min. The washed precipitates were dissolved in 4 drops of water plus 4 drops of 0.5N-NaOH, and the solutions transferred to brass sample plates (containing kaolin). Each tube was washed out three times with 3 drops of water each time, and the washings added to the sample plates, which were then placed on a warm water bath until dry. A control experiment, with exactly the same technique, was carried out with normal rabbit serum added to the antigen solutions. Any radioactivity observed in the control, which was appreciable only with high concentrations of antigen, was subtracted from the value obtained for the corresponding mixture of antigen and antiserum.

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

Radioactivity measurements. These were made with the Geiger-Müller counter system described by Banks (1946). Owing to the much greater penetrating power of the radiations from P^{32} as compared with those from S^{35} , however, it was not found necessary to incinerate the materials before they were 'counted'. The plates were of the usual pattern, but were fitted with disks of 2 cm. diameter to improve the geometrical arrangements of the sample relative to the counter window. To facilitate the spreading and drying, about 50 mg. of kaolin were first suspended in a few drops of 50% ethanol and this suspension evaporated on the plate. The solution to be 'counted' (up to 0.5 ml.) was dropped on the kaolin pad and the plate heated over a warm water bath; the dried sample was protected against damage by adding 10-15 drops of 1:50 collodion in ethanol-ether mixture and was then redried.

This simple evaporation method for the preparation of samples for P^{32} determinations on serological precipitates and other organic material containing the radio-isotope greatly facilitates the determinations. It is desirable that, where this method is used, checks be made by incinerating some of the material and separating the phosphate as magnesium ammonium phosphate for radioactivity determinations. This control procedure has been adopted in the work described in this paper and in all cases the simple 'evaporation' method has given essentially the same result as the digestion or incineration method.

RESULTS

The excretion of P^{32} in various fractions of the eggs laid by hens injected with P^{32} -containing phosphate

The total radioactivities of the shell, whole yolks, and lipid and lipovitellin fractions of the eggs laid by the two hens during the month following the in-

Fig. 1. Radioactivity of the intact egg (i.e. shell) and the yolk of eggs from hens injected with phosphate containing P^{32} . The values given in these figures are not absolute values, but merely give the relative activities of the shell (or yolk) from day to day (see text).

jection of 50 μC of P^{32} into each hen are given in Figs. 1 and 2. Owing to normal variations between individual eggs, particularly those laid by different hens, the points do not always lie on a smooth curve; but it is quite evident that the radioactivity of the vitellin and lipid fractions of the yolks reached a maximum about the 4th day after the injection,

remaining approximately constant for a further 6 or 7 days, and then decreasing rapidly. For most of our subsequent immunological work, eggs laid 4–11 days after the injection of P^{32} have been used for the preparation of radio-vitellin and radio-lecithin. At the maximum, each egg contained in the lipovitellin plus lipid about 1% of the total P^{32} injected, with about two thirds of this in the

Fig. 2. Radioactivity of the yolk lipids and lipovitellin of eggs from hens injected with phosphate containing P^{32} . The values are expressed as activity/single egg. From the 1st to 12th day, each individual egg was examined; thereafter the eggs were grouped in batches of seven. Hen no. 815 —□—. Hen no. 816 —○—. Mixed yolks (815 + 816) —×—.

lipid fraction. The activities given for the shells and the whole yolks are, as pointed out above, purely arbitrary values and have only relative significance.

The determination of P^{32} in vitellin-anti-vitellin precipitates

Table 1 gives the results of determinations of the amount of antigen in the precipitates obtained by treating 0.20 ml. samples of a lipovitellin antiserum

Table 1. Radio-P content of precipitates with 'radio'-vitellin and rabbit anti-vitellin serum

(0.20 ml. of antiserum + 0.20 ml. of 20% (w/v) NaCl were added to 2.0 ml. of 'soluble' radio-vitellin in 10% (w/v) NaCl. Precipitates were centrifuged after 3 hr. at 37°. Control: normal rabbit serum instead of antiserum.)

Concentration of antigen (% protein)	Radio-P in 2 ml. antigen ($\mu\text{g.}$)	Radio-P precipitated			
		Rabbit antiserum* ($\mu\text{g.}$)	Control serum ($\mu\text{g.}$)	Average minus control ($\mu\text{g.}$)	As % of total antigen P
2.3	942	9.45, 12.4	13.4	0.0	0.0
0.37	157	30.9, 32.6	0.6	31.1	20
0.13	52.3	18.3, 18.3	0.5	17.8	34
0.042	17.4	6.34, 7.02	0.4	6.3	36
0.014	5.81	2.2, 2.1	0.0	2.1	37
0.0046	1.94	0.85, 0.61	0.0	0.7	37

* Duplicate experiments.

(no. 457) with 2.0 ml. samples of solutions containing radioactive lipovitellin. (Both lipovitellin and 'insoluble' vitellin injections produced antisera capable of precipitating lipovitellin.) It can be seen that, with the lower concentrations of antigen, approximately the same fraction (about 37 %) of the total P^{32} present in the antigen solution was pre-

conditions of our experiments. From the results of this experiment (Fig. 3) it will be seen that, with the lowest concentrations of antigen, there was again precipitation of about 40 % of the antigen P. Where there was the maximum amount of P^{32} in the precipitate, this represented about 25 % of the P^{32} in the antigen solution used.

Fig. 3. P^{32} content of lipovitellin-antilipovitellin precipitates. A, percentage of total antigen P precipitated. B, total P precipitated (μ g.). Antigen: 2.0 ml. radio-lipovitellin (P/N ratio = 0.14) in 10 % NaCl. Antiserum: 0.2 ml. rabbit antiserum to lipovitellin + 0.2 ml. 20 % NaCl. Precipitates: centrifuged after 3 hr. at 37° and washed once with 10 % NaCl.

cipitated in each case, down to extremely low concentrations of antigen. With moderate excess of antigen, the percentage of antigen P precipitated was less, while with a large excess none of the P^{32} was precipitated, thus confirming the visual observation in the precipitin test mixtures.

In a further experiment a much smaller range of antigen concentration was covered, involving the use of antigen (radio-lipovitellin) solutions varying in strength from 0.05 to 0.74 %. This range covers the points of maximum precipitation and extends into the excess-antigen inhibition zone, under the

The part played by lipids in the lipovitellin-antivitellin precipitin reaction

Since vitellin preparations, particularly the 'soluble' variety, contain considerable amounts of phospholipins, a few experiments have been made to determine whether these take part in the precipitin reaction. Varying amounts of radio-lipovitellin were added to measured volumes of anti-vitellin serum, four tubes being set up for each antigen dilution. After the mixture had been incubated and centrifuged, two tubes of each mixture were extracted three times with an ethanol-ether mixture to remove lipid matter. P^{32} determinations were made on the unextracted and the extracted precipitates and on the ethanol-ether extracts. These results (Table 2) showed that 70–80 % of the P^{32} in these specific precipitates was of phospholipid type (i.e. extractable with ethanol-ether). Since the lipovitellin preparations A and B used as antigens contained only 47 and 41 % respectively of their total P^{32} as phospholipin, there appears to have been preferential precipitation of much of the phosphatide by the antiserum. Parallel control tests made with an unrelated precipitin reaction (human serum plus anti-human serum) carried out in the presence of radio-lipovitellin gave precipitates containing no significant amount of P^{32} . It is concluded, therefore, that the preferential precipitation of phospholipin in the lipovitellin-antivitellin precipitates is not simply due to the mechanical carrying down of lipid by the precipitate.

Table 2. *The lipid P of lipovitellin-antivitellin precipitates*

(Precipitin reactions as in Table 1. Some of the precipitates were extracted three times at room temperature with 5 ml. of 7 : 3 ethanol-ether for 30 min. each time. The extracted precipitates were dissolved in dilute NaOH, and the ethanol-ether extracts were concentrated to small bulk. Both solutions were then transferred to sample plates for P^{32} determinations.)

Exp.	Concentration of antigen (%)	Antigen* (μ g./2 ml.)	Radio-P content		
			Lipovitellin-antivitellin precipitate		
			Total (μ g.)	Extractable by ethanol-ether	
				(μ g.)	As % of precipitated P
1	0.377	157.5	37.7	26.9	71
	0.251	104.7	34.0	24.7	73
2	0.322	152.4	7.4	5.8	78
	0.214	101.6	11.3	9.0	80
	0.161	76.2	10.2	7.9	78

* 47 % of the P^{32} of the lipovitellin (preparation A) used as antigen in Exp. 1 was extractable with ethanol-ether, and the corresponding value for preparation B (Exp. 2) was 41 %. A and B had P/N ratios of 0.14 and 0.158, respectively.

In other experiments radio-ovolecithin has been added to mixtures of non-radio-lipovitellin and anti-vitellin. The specific precipitates formed in these mixtures contained very appreciable amounts of radio P. Further experiments are being made to determine whether this is simply due to interchange of lecithin between the solution and the antigen, or whether it is due to the direct combination of the radio-lecithin with the antivittellin or with the antigen-antibody complexes.

DISCUSSION

For the preparation of a 'labelled' protein which can be used as an antigen, the injection of P^{32} into hens and the separation of P^{32} -containing vitellin from the eggs laid by these hens has proved very satisfactory, with a reasonably good utilization of P^{32} . The injection of P^{32} is known to lead to the early appearance of the radio isotope in the various P-containing compounds of the hen and its eggs (Hevesy & Hahn, 1938; Aten, 1939; Chargaff, 1942b). In our experiments, the shell of the egg laid the day after the injection was highly radioactive, but that laid on the second day had only about half this activity; subsequently, the radioactivity of the shells fell to a relatively low level (Fig. 1). The whites of the eggs showed very little radioactivity, particularly after the first day or two, but the P^{32} content of the yolk lipids and the lipovitellin increased fairly rapidly up to a maximum at about 4 days after the injection, and then decreased slowly (Fig. 2).

These results indicate that the eggs laid 4-11 days after the injection of P^{32} into the hen serve best as sources of P^{32} -containing vitellin and lecithin. In one experiment, for example, about 2% of the injected P^{32} was present in the lipovitellin and a little over 4% in the yolk-lipids of the eggs laid during this period. Labelled vitellin and lecithin can thus be readily obtained with a specific radioactivity adequate for many immunological purposes, including the use of these compounds in micro-precipitin tests and for some *in vivo* immunity experiments. In many of the precipitin tests described in this paper, rather large amounts of antigen solution were used, because of the relatively small amount of P^{32} available to us, but it is possible, by injecting more P^{32} and by adopting a special micro-precipitin-test technique, to make reliable P^{32} determinations on precipitates from considerably less than 0.10 ml. of antigen. Although we used relatively large volumes of antigen solution for the tests, it should be remembered that some of our precipitates which gave 'counts' which could be measured accurately, were obtained from less than 0.05 ml. of the stock antigen solution (2.3% radio-lipovitellin in 10% NaCl solution).

Lipovitellin is not an ideal protein complex for serological investigations, partly because of its re-

lative insolubility and partly because it contains a very considerable amount of bound phosphatide. The first difficulty can be overcome by adding NaCl, to give a concentration of 5-10% (w/v), to all the solutions used for the precipitin tests; but the second problem cannot be solved so easily. The extraction of the firmly bound phosphatide with hot ethanol or with ethanol-ether mixtures in the cold renders the vitellin almost insoluble in water or salt solutions. It is then practically useless for precipitin tests, although it might be satisfactory for complement-fixation tests.

The immunological properties of vitellin have been studied by several authors. Wells (1909, 1911) showed, by anaphylactic experiments, that it is antigenic and that it is serologically distinguishable from ovomucoid and ovalbumin. Emmerich (1913) and Seng (1913) investigated the serological cross reactions between the yolk proteins of the hen's egg and those of the eggs of other birds, but as no separation of the vitellin was carried out, many of their results might well have been due to the other proteins present; hen egg-yolk livetin, for example, is antigenic, and immunological tests show that it is closely related to hen serum globulin (Jukes & Kay, 1932). Roepke & Bushnell (1936), using 'soluble' vitellin, obtained antisera which gave specific precipitation, and they were able to show that the serum of laying hens contains significant amounts of a protein similar to vitellin.

In our experiments immunization was effected with both the 'soluble' and the 'insoluble' vitellin, and both preparations produced good antisera in rabbits: since both these antisera gave precipitates with 'soluble' vitellin it is concluded that at least some of the reactive groupings in the antibodies are directed against groups of the protein molecule. Whether or not these antibodies can react with the lipid groups of lipovitellin is not known. The yolk phosphatides have been carefully studied in more recent years by Chargaff (1942 a, b; cf. also Chargaff, Ziff & Rittenberg, 1942) who found that removal of all the uncombined lipids with ether and repeated dialysis yielded a lipovitellin containing about 18% of phosphatide and a total P content of 1.5% (compared with 1.0% in the ethanol-extracted product). Our 'soluble' vitellin preparations contained about the same percentage of phosphatide (15-22%) as did the preparations of Chargaff, accounting for about half the P of the 'soluble' vitellin.

The specific precipitates obtained in our experiments contained considerable amounts of phosphatide, more in fact than did the lipovitellin used as antigen. In one experiment (e.g. Exp. 2, Table 2) about 80% of the P^{32} of the precipitates was due to phospholipin, as against a figure of 41% for the antigen. The combination (or perhaps adsorption) of the phosphatide by the antigen-antibody complex

appears to be specific since a heavy precipitate formed between human serum proteins and their antibodies in the presence of radio-lipovitellin did not carry down any significant amount of phospholipin. It seems possible, therefore, that the lipovitellin antibodies have groups capable of reacting specifically with ovophosphatide. Precipitation might only occur in these mixtures when the antibodies have also reacted with the protein vitellin or with lipovitellin. It is however difficult to explain how the antibodies detach phosphatide from a lipoprotein complex, from which the phosphatide cannot be removed by ether extraction.

The nature of the linkage between ovophosphatide and the protein vitellin is not at all clear, and it would be of interest to know whether there is any significant serological difference between vitellin and lipovitellin. Removal of the ether-soluble substances from serum does not affect the true antigenic properties of the serum, i.e. its power to produce antibodies on injection, but specific precipitation does not occur when both antigen and antiserum are fully extracted with cold ethanol-ether (Hartley, 1925). Other specific precipitates vary in their lipid content, for whereas Horsfall & Goodner (1936) found 4-51% of lipid in their pneumococcus polysaccharide precipitates, Marrack & Smith (1931) were unable to find any lipid in horse serum pseudoglobulin antigen-antibody precipitates.

The experiments described in the present paper were designed to indicate how a radio-isotopic tracer might be of value in one branch of immunochemistry. The results show that the tracer can be determined quite readily in precipitates obtained in ordinary precipitin tests. In these experiments no attempt was made to get maximum precipitation in

all cases, e.g. by keeping the antigen-antibody mixtures in the refrigerator for several days, nor were any special efforts made in most of these experiments to purify the vitellin used as antigen, for it was not our desire to remove the firmly bound lipid from the vitellin. The results of other experiments, with fully extracted lipid-free vitellin, will be reported later.

SUMMARY

1. P^{32} -containing vitellin prepared from eggs laid by hens injected with P^{32} -containing phosphate has been used for immunological investigations.

2. P^{32} determinations have been made on precipitates obtained in ordinary precipitin tests to determine the amount of antigen in vitellin-antivitellin precipitates.

3. This isotopic tracer method, together with total-N determinations, will readily allow antigen/antibody ratios to be determined. Other possible uses of radio-isotopes for the study of immunochemistry are indicated.

4. Lipovitellin-antivitellin precipitates contained a much higher ratio of phosphatide P^{32} /protein P^{32} than did the lipovitellin antigens. This preferential carrying down of phosphatide by the precipitate is specific, and it does not occur in precipitin reactions unrelated to vitellin, e.g. in the precipitation of human serum proteins by their antibodies.

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