REFERENCES

- Association of Official Agricultural Chemists (1947). *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 2nd ed. Washington.
- Bradley, W. B., Eppson, H. F. & Beath, O. A. (1939). *J. Amer. vet. med. Ass.* **94**, 541.
- Bradley, W. B., Eppson, H. F. & Beath, O. A. (1940). *J. Amer. vet. med. Ass.* **96**, 41.
- Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood and Son.
- Evelyn, K. A. & Malloy, H. T. (1938). *J. biol. chem.* **126**, 655.
- Lees, H. & Quastel, J. H. (1946). *Biochem. J.* **40**, 803.
- McDonald, I. W. (1948). *Biochem. J.* **42**, 584.
- McDonald, I. W. (1949). Personal communication.
- Newsom, I. E., Stout, E. N., Thorp, E., Barber, C. W. & Groth, A. H. (1937). *J. Amer. vet. med. Ass.* **90**, 56.
- Phillipson, A. T. & Innes, J. R. M. (1939). *Quart. J. exp. Physiol.* **29**, 333.
- Rimington, C. & Quin, J. I. (1933). *Onderstepoort J. vet. Sci.* **1**, 469.
- Russell, F. C. (1944). *Tech. Commun. Bur. Anim. Nutrit., Aberd.*, **15**, 87.
- Sapiro, M. L., Hoflund, S., Clark, R. & Quin, J. I. (1949). *Onderstepoort J. vet. Sci.* **22**, 357.

The Use of Radioactive Isotopes in Immunological Investigations

5. QUANTITATIVE ASPECTS OF THE ANTIBODY-ANTIGEN REACTION

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The reaction between antigen and antibody which results in precipitation has been studied quantitatively by Heidelberger, Marrack, Haurowitz, Kabat, Hooker and Boyd, and many other investigators (see reviews by Heidelberger, 1939, 1946; Marrack, 1938; Boyd, 1947; Kabat & Mayer, 1948; Wormall, 1948). These investigations have been based mainly on the determination of small amounts of nitrogen in the precipitates. The amount of antibody can be readily calculated from these determinations if the antigen contains little or no nitrogen (e.g. polysaccharides) or alternatively, the amounts of both antigen and antibody can be calculated, if the antigen contains some special group or element (e.g. dyestuff, iron, copper, etc.) which can be determined separately. Few natural proteins, however, contain a suitable label, and the introduction of foreign groups, leading to the production of unnatural proteins or protein complexes, is frequently undesirable. When unlabelled protein antigens are used, the analysis of the specific precipitates for both antigen and antibody is complicated, and may sometimes be impossible.

We have used antigens and antibodies labelled with radioactive isotopes to study the precipitin test and certain other serological reactions. At first our choice of antigen was limited to biologically prepared labelled proteins such as lipovitellin and vitellin (Francis & Wormall, 1948), and chemically altered proteins such as phosphorylated or mustard gas sulphone-treated proteins containing appreciable quantities of introduced phosphorus or sulphur

(Bournsnel, Dewey, Francis & Wormall, 1947; Banks, Bournsnel, Dewey, Francis, Tupper & Wormall, 1948). With an improved supply of certain carrier-free radio-isotopes it later became possible, however, to use such small amounts of the element that the protein molecule could be adequately labelled without significant change in the properties of the protein other than the acquirement of radio-activity. The experiments described here were carried out in order (a) to obtain information about the best methods for labelling protein antigens and antibodies with radio-isotopes for serological investigations, and (b) to use these labelled compounds for quantitative studies on certain precipitin reactions. In most of these experiments chemically altered protein antigens have been used, e.g. those labelled with 8–12 % radio-iodine; but where antibodies have been labelled with ^{131}I this has been effected with small amounts of radio-iodine of high specific activity. Preliminary notes of a few of these investigations have been published elsewhere (Bournsnel *et al.* 1947; Banks, Francis, Mulligan & Wormall, 1949, 1950).

EXPERIMENTAL

Methods

Precipitin reactions. Since we usually varied the technique of these tests to suit the particular experiment undertaken, only the general details will be given here; exact details, where they are required, are given in the appropriate tables or parts of the text. The antigen and antibody solutions, plus

0.9% NaCl solution to bring to a standard volume, were mixed in tapered 15 ml. centrifuge tubes, and the mixtures kept at 37° for 3 hr. (or other specified time) and then overnight or for 2–4 days in the refrigerator (0–4°). The centrifuged precipitate, usually after it had been washed twice with small volumes of cold 0.9% NaCl, was dissolved in a small volume of dilute (e.g. 0.5N) NaOH, and the solution diluted sufficiently to permit samples to be taken for all the radioactivity and chemical determinations. Since lipovitellin is almost insoluble in 0.9% NaCl solution, all precipitin tests with this antigen were carried out with mixtures containing about 10% (w/v) NaCl.

The washing of the antigen-antibody precipitates with 0.9% NaCl, to remove 'non-specific' proteins, was carried out in most of these experiments, for it has been shown that the amount of N in the specific precipitate is not thereby altered (Heidelberger & Kendall, 1932). The time required for complete precipitation of antigen-antibody complexes varies according to the system used and the experimental conditions, but it is generally agreed that with strong rabbit antisera precipitation is essentially complete in 1–2 hr. at 37° (see Kabat & Mayer, 1948, p. 36).

Total nitrogen determinations. These were made by the micro-Kjeldahl method, with the NH_3 distilled, in the apparatus described by Markham (1942), into a boric acid solution containing a methyl red-methylene blue indicator. The final titration was made with $\text{N}/70\text{-HCl}$, using an 'Aglar' Micrometer syringe (Burroughs Wellcome and Co.) and with a stream of N_2 bubbling through the boric acid mixture. The method is generally similar to that described by Kabat & Mayer (1948). With this technique less than 100 μg . N can be determined with an accuracy of $\pm 1\text{--}2\ \mu\text{g}$.

When precipitates containing a radioactive antigen and unlabelled antibodies are analysed, the radioactivity determination enables the amount of antigen to be calculated, and the total N less the antigen N gives the amount of antibody N. No allowance has been made in these experiments for any complement N carried down by the specific precipitate, for this amount is very small; furthermore, all the antisera used were filtered through a Berkefeld filter, which removes some, at least, of the components of complement.

Phosphorus determinations. Total P was determined by King's (1932) modification of the method of Fiske & Subbarow (1925). 60–70% of the P of most lipovitellin preparations can be extracted by treatment with an ethanol-ether mixture (7:3 by vol.) (Francis & Wormall, 1950), and for the purposes of this paper this fraction is called lipid P, and that not extracted, the vitellin P.

Total iodine determinations. These were made by the method of Shahrokh (1943). Under the conditions of our experiments we have found this method satisfactory for the determination of 10–100 μg . of I_2 , with an accuracy of $\pm 1\ \mu\text{g}$.

Calculation of molecular ratios. It was not practicable to determine the molecular weights of the iodinated proteins

and other antigens and antibodies used in the tests. In calculating the molecular ratios recorded in this paper, the following molecular weights have been used: ovalbumin, 40,500 (Svedberg & Eriksson-Quensel, 1935–6); antibodies in rabbit sera, 165,000 (for the references, see Kabat & Mayer, 1948, p. 168); human serum globulins, 165,000. Similar values, increased to allow for the amount of I_2 introduced, have been used for the iodinated proteins. The N content of ovalbumin has been taken as 15.76% (Chibnall, Rees & Williams, 1943) and that of antibodies and all other proteins studied as 16%.

Radioactivity determinations. The Geiger-Müller counter system described by Banks (1946) was used for these determinations. Side window or bell-type end window counters have been used for most of the ^{131}I measurements and generally for determinations on solid preparations. The 'liquid counter' described by Veall (1948) was used on solutions and values obtained with ethanol-ether solutions were corrected for specific gravity differences.

Determination of ^{131}I and ^{32}P in the same precipitate. In some of our experiments we have used ^{32}P to label the antigen (lipovitellin) and ^{131}I to label the antibodies, and it was necessary to devise some method of determining each radioisotope in a mixture containing both. Since differential counting methods, using β - and γ -ray counters, proved unsatisfactory, the determinations were effected by measuring the total radioactivity of samples before and after removing the ^{131}I .

A sample (1 ml.) of the solution of the specific precipitate was heated with KClO_3 (about 0.5 g.) and approx. 20N- H_2SO_4 (2 ml.), to convert all the I_2 to iodate. After addition of 0.1N- KIO_3 (1 ml.) as carrier, the I_2 was liberated by adding excess of 1% KI (2.0 ml.) and removed by two extractions with CHCl_3 . Since the remaining KI was radioactive, owing to exchange between iodide ion and I_2 , it was then removed by the addition of a slight excess of 0.1N- KIO_3 and two further extractions with CHCl_3 . No significant further exchange of ^{131}I apparently takes place at this stage, for there is no interchange between radioactive I_2 and non-radioactive iodate under these conditions (cf. also Perlman, Chaikoff & Morton, 1941). The radioactivity of the de-iodinated material is a measure of the ^{32}P , and the difference between this activity and the activity of the original material is a measure of the ^{131}I . A small correction (7–10 counts/min. in the liquid counter) had to be made in the values for the de-iodinated material, to compensate for the natural radioactivity of the ^{40}K of the KClO_3 . When the KClO_3 was replaced by NaClO_3 there was considerable spurting during the incineration, and it was decided, therefore, to continue to use KClO_3 .

Determinations made with mixtures of proteins, some labelled with ^{32}P and some with ^{131}I , gave satisfactory results. The agreement between the observed and theoretical values was very good for

Table 1. Determination of ^{32}P and ^{131}I in mixtures of proteins labelled with the two isotopes

Mixture	^{32}P		^{131}I	
	Added (counts/min.)	Found (counts/min.)	Added (counts/min.)	Found (counts/min.)
A	414	427	101	97
B	372	369	88	92
C	597	599	279	301

^{32}P , but not quite so good for ^{131}I , possibly because the latter determination is made by difference. The results of a few recovery experiments of this type, with mixtures of ^{32}P -containing lipovitellin and ^{131}I -containing rabbit serum proteins, are given in Table 1.

MATERIALS

Antigens

All the antigens were used immediately after preparation or were treated with 0.01% merthiolate and stored in a refrigerator.

Ovalbumin. Solutions of thrice crystallized hen ovalbumin in 0.9% (w/v) NaCl were used.

Serum globulins. The mixed serum globulins were precipitated by addition of 1 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The precipitate was dissolved in 0.9% NaCl (1 vol.) and reprecipitated with $(\text{NH}_4)_2\text{SO}_4$; the precipitate was then dialysed against 0.9% NaCl at 0–5° until the external solution gave no reaction with Nessler's reagent. The horse serum used for a few of the early experiments was 'Normal Horse serum no. 2' (Burroughs Wellcome & Co.). We subsequently learned that this preparation is heated at 58° for about 2 hr. before issue, and in all our later experiments we used fresh unheated normal horse serum.

Lipovitellin. ^{32}P -containing lipovitellin was prepared, as described by Francis & Wormald (1948), from the eggs laid by hens injected with ^{32}P -containing inorganic phosphate; the final solution contained 125.5 mg. total P and 48 mg. vitellin P/100 ml. A similar non-radioactive preparation was used for immunization.

Iodinated proteins (general). Various iodination methods were used, but nearly all involved iodination in ammoniacal solution by a modification (Wormall, 1930) of the method of Blum & Strauss (1923). This method, which is particularly suitable for immunological investigations, is not completely satisfactory for use with radioactive iodine, since it involves loss of ^{131}I as iodide. In earlier experiments, not reported here, where it was necessary to obtain the maximum utilization of ^{131}I , this loss was avoided by using a solution of I_2 in ether.

Iodinated ovalbumin (containing ^{131}I). KI (0.4 ml. of 10% solution) was added to 150 μc . of ^{131}I as NaI solution (0.1 ml.) followed by 0.1 N-KIO₃ (1.43 ml.), and the I_2 was liberated by adding 0.1 N-HCl (1.43 ml.); the KI present was sufficient to keep the liberated I_2 in solution. This solution was added, dropwise with shaking after each addition, to a mixture of a 2.5% (w/v) solution of ovalbumin (2 ml.) and 5 N-NH₃ (1 ml.) at room temperature. The iodinated ovalbumin was precipitated by adding acetic acid (5 N followed by N) to give maximum precipitation. The precipitate was dissolved in 0.9% NaCl at pH 7.5, and three further precipitations with acetic acid were carried out, the final supernatant solution being then free from detectable I_2 or iodide. This iodinated ovalbumin solution (5 ml.) in 0.9% NaCl, with 2 N-Na₂CO₃ added to bring the pH to 7.5, contained 135 mg. N and 74.6 mg. I/100 ml.; these values correspond to an iodine content of 8.1% for the iodinated ovalbumin. About one-ninth of the ^{131}I used for the iodination was present in the iodinated ovalbumin.

Iodinated human serum globulins (containing ^{131}I). The method used was as described for the preparation of ^{131}I -containing iodinated ovalbumin. The final solution, in 0.9%

NaCl at pH 7.5, contained 110 mg. N and 69.4 mg. I/100 ml.; this corresponds to an average I content of 9.2% for the iodinated globulins.

Iodinated ovalbumin (non-radioactive). A solution of 0.1 N- I_2 in KI (7 ml.) was gradually added to a 2.5% solution of ovalbumin (10 ml.) plus 5 ml. 5 N-NH₃, free I_2 being detectable 5 min. after the last addition of the I_2 solution. The iodinated ovalbumin was separated and purified by precipitation with dilute acetic acid and resolution in 0.9% NaCl at pH 7.5 (four precipitations in all) as described above. The final product contained 7.3% I (based on I and N determinations).

Iodinated horse serum globulins (non-radioactive). A solution of the mixed globulins was iodinated by the method described for non-radioactive iodinated ovalbumin. Based on N and I determinations the iodinated globulins contained about 10% of I.

Iodinated rabbit serum proteins (non-radioactive). For a few precipitin tests we used, as test antigen, the iodinated mixed albumins and globulins of rabbit serum. Serum (7.5 ml.) was mixed with water (7.5 ml.) and 5 N-NH₃ (7.5 ml.) and the proteins were iodinated, and the products precipitated, as described for non-radioactive iodinated ovalbumin. The final solution, in 0.9% NaCl and at pH 7.5, contained 299 mg. N and 269 mg. I/100 ml., corresponding to an average I content of 12.6% for the mixed iodinated serum proteins.

Antibodies

All the antisera used in these experiments were obtained by immunizing four or five rabbits with each antigen (lipovitellin or iodinated horse serum globulin). A few antisera were prepared by injecting the antigen intraperitoneally at intervals of 8–10 days, but most of the best sera were obtained in response to intramuscular injections of the alum-precipitated proteins (Proom, 1943). Potent antisera were frequently obtained 9–10 days after the first injection of the alum-precipitated antigen, and most of the remaining rabbits of these groups gave good antibody response after the second or third injection. For some experiments the serum of one rabbit was used, but the antisera from each group of rabbits were usually pooled.

The antisera were filtered through Berkefeld filters, and kept either in sterile tubes at 0–4° or in sealed tubes after freeze-drying in the apparatus described by Greaves (1946). No significant difference was observed between the serological properties of the filtered and those of the freeze-dried antisera to iodinated proteins. But since there are differences in the case of preserved antisera to lipovitellin (Francis & Wormald, 1950), it is considered advisable to note here the mode of preservation of each of the antisera used in the experiments referred to in the tables and figures of this paper: namely, filtered and stored at 0–4° (Tables 2 and 3, Figs. 1 and 2), filtered and freeze-dried (Table 4).

Globulin fraction of antiserum. The total globulins of some antisera were separated by adding 1 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The precipitate was dissolved in 0.9% NaCl, reprecipitated by $(\text{NH}_4)_2\text{SO}_4$, and dialysed against 0.9% NaCl at 0–4°. Some of these globulin fractions were iodinated as described below.

Iodinated antibodies. Preliminary tests with ordinary I_2 indicated that introduction of 1% I_2 into various precipitating antibodies caused no serious reduction in their capacity to precipitate the corresponding antigen; indeed this precipitating power is not completely abolished when

3% or sometimes appreciably more I_2 is introduced. Quantitative experiments with ^{131}I , to be reported later, have fully confirmed this observation. For tracer experiments, when radio-iodine of high specific activity is available, very much smaller amounts of I_2 are adequate, but for reasons given later we have usually used sufficient I_2 to introduce 0.7–1.0% into the antibody proteins. The iodination was effected in the presence of NH_3 , but in order to avoid destruction of antibody by this base, the amount of NH_3 used was considerably less than that used in the iodination of antigens.

A solution of I_2 in KI (19 mg. free I_2) containing 121 μc . ^{131}I was prepared as described above. This solution was then added, dropwise and with gentle shaking, to a mixture of the total globulins (22.5 ml. of solution containing 0.83 g. of protein) of an antilipovitellin serum and 5 N- NH_3 (4.0 ml.). The mixture was kept at room temperature for 15 min. after the addition of all the I_2 solution, and 5 N-acetic acid was then added to bring the pH to 7.5. The solution was dialysed against 0.9% NaCl at 0–4°, with a glass marble in the dialysing sac to permit mixing of the contents at intervals. The dialysis was continued until all diffusible radioactive material had been removed, and the solution of iodinated antibody globulin, diluted to 45 ml. with 0.9% NaCl, was used for the experiments described in Table 4. These labelled globulins contained 0.88% I , and gave a count of about 800/min./mg. of protein N in a liquid counter (or about ten times as much with a bell-type counter) when the analyses of the precipitates were carried out.

In later experiments, using the iodinated γ -globulin fraction of an antiserum, it has been found that complete removal of loosely attached ^{131}I can be more satisfactorily achieved by dialysing overnight and then precipitating the proteins several times with 12% (w/v) Na_2SO_4 .

RESULTS

Iodinated protein antigen-antibody precipitates

The reaction between several iodinated protein antigens and antibodies to iodinated horse serum globulins was studied over a wide range of antigen concentration. In the experiments recorded in Figs. 1–3 the antigens were labelled with ^{131}I and the amount of antibody in the precipitate was calculated from the total N content, allowing for the amount of antigen N present. The iodinated protein antigens used in the precipitin tests were of different origin from those used for immunization, and this use of heterologous antigens makes it possible for the precipitin reaction to be confined largely to a reaction between the antibody and those parts of the antigen molecule containing diiodotyrosine.

Mixtures of antigen (iodinated ovalbumin or iodinated human serum globulin, both containing ^{131}I) and an antiserum to iodinated horse serum globulin (1–2 ml.) were made up with 0.9% NaCl to 5 or 6 ml. (globulin and albumin series respectively), and were kept at 37° for 3 hr. and subsequently at 0–5° for 90 hr. The centrifuged precipitates were washed three times with 2 ml. ice-cold 0.9% NaCl, dissolved in water plus a few drops 0.5 N-NaOH, and made up to 3 ml. Duplicate determinations of total N and radio-iodine were made on 1 and 0.25 ml. samples respectively.

The results of typical experiments (Figs. 1 and 2) were generally similar to those recorded for homologous antigen-antibody systems such as that between ovalbumin and anti-ovalbumin (cf. reviews by Boyd, 1947, and Kabat & Mayer, 1948). The maximum amount of antibody precipitated by the iodinated human serum globulin was, as was anticipated, considerably greater than that occurring with

Fig. 1. Composition of specific precipitates obtained with iodinated human serum globulin and a rabbit antiserum to iodinated horse serum globulin. The broken lines in this figure and in Fig. 2 correspond to complete precipitation of the antigen.

Fig. 2. Composition of specific precipitates obtained with iodinated ovalbumin and a rabbit antiserum to iodinated horse serum globulin.

the less closely related iodinated ovalbumin. Moreover, except possibly in regions of large antibody excess, the whole of the added heterologous antigen was not precipitated by the antibodies. The molecular ratio of antibody to antigen in the precipitates obtained with iodinated human serum globulin ranged from 15 in the region of antibody excess to 2.5 in the zone of antigen excess. The corresponding range for precipitates obtained with iodinated ovalbumin as antigen was 4.5–1.5 (Fig. 3).

For comparison typical results of some closely related experiments with a non-radioactive iodinated ovalbumin are given in Table 2. To allow a complete

duplicate analysis of the precipitates, it was necessary to use more antiserum and antigen in these experiments than was needed in the experiments with the radioactive antigen; e.g. in the experiment

Fig. 3. Antibody-antigen molecular ratios in specific precipitates obtained with a rabbit antiserum to iodinated horse serum globulin. With all mixtures corresponding to points to the left of the arrow, excess antibody was detectable in the supernatant solution.

described in Table 2, 4–8 ml. of antiserum were added to each tube, as compared with 2 ml. with the ^{131}I -containing iodinated ovalbumin. The molecular ratio of antibody to antigen in the precipitates referred to in Table 2 ranged from 5.0 in the region of antibody excess to 1.9 in the zone of antigen excess.

In order to eliminate as completely as possible the serological effect of the original protein used for the preparation of the immunizing iodinated protein antigen, precipitin tests similar to those described above were made with iodinated mixed rabbit serum

proteins as the test antigen. The specific reaction in this case was probably confined entirely to one between the antibodies and the 3:5-diiodotyrosine groups of the antigen. Although this experiment was not made with radio-isotope-labelled reagents the results are included here (Table 3), since there are little quantitative data available in relation to serological precipitates with labelled rabbit serum proteins used as the test antigen in precipitin reactions with rabbit antisera. In view of the fact that the test antigen was a mixture of iodinated globulins and albumins, it would be unwise to attach great significance to antibody/antigen molecular ratios for the precipitates obtained, but it is interesting to note that if an arbitrary average molecular weight of 100,000 is assumed for the proteins of the test antigen, the molecular ratio in the precipitates ranged from 4.4 (antigen excess zone) to 12.6 (antibody excess).

Composition of lipovitellin-antilipovitellin precipitates

In several experiments we separately labelled the antigen and antibody with different radioisotopes. In one series of experiments we used ^{32}P -containing lipovitellin as the antigen, and the corresponding antibodies were labelled with ^{131}I . By using radioiodine of fairly high specific activity we were able to avoid significant loss of serological activity of the antibodies. Since the antigen contained lipid P as well as protein (vitellin) P, the serological precipitates were analysed for these two P fractions, as well as for antibody. Preliminary tests showed that ^{131}I could be quantitatively removed from the antigen-antibody complexes without loss of ^{32}P , as is described earlier in this paper. Briefly, the radioactivity measurements on each precipitate involved determinations on (a) the material extracted by ethanol-ether (lipid P), (b) the extracted precipitate (vitellin

Table 2. Composition of precipitates obtained with iodinated ovalbumin and antisera to iodinated horse serum globulin

(Mixtures of antigen (iodinated ovalbumin) solution and antiserum (4 ml. in tubes 1 and 2; 8 ml. in tubes 3, 4 and 5) were made up with 0.9% NaCl to 9 ml. total volume in each case, incubated at 37° for 3 hr. and kept at 0–5° for a further 90 hr. The centrifuged precipitates were washed twice with 0.9% NaCl (6 drops each time), dissolved in water plus a few drops 0.5N-NaOH, and made up to 15 ml. Duplicate total N and I determinations were made on 1 and 6 ml. samples respectively, of these solutions.)

Tube no.	Antigen added		Tests† on supernatant solution		Composition of precipitate				
	N* (μg.)	I* (μg.)	Antigen present	Antibody present	Total N (μg.)	Total I (μg.)	Antigen N (μg.)	Antibody N (μg.)	Mol. ratio antibody/antigen
1	47.8	23.6	tr.	0	272	15.2	30.7	241	1.9
2	23.9	11.8	tr.	f.tr.	242	8.4	17.0	225	3.2
3	11.9	5.9	0	±	151	5.1	10.3	141	3.4
4	9.55	4.72	0	+	149	3.9	7.9	141	4.4
5	7.96	3.94	0	+	122	2.8	5.7	116	5.0

* All the N and I values in this table are expressed as μg./ml. of antiserum used.

† Tested by the addition of antiserum and antigen to separate samples; 0, none detectable; f.tr., faint trace; tr., trace; ±, moderate and +, strong, indicate the extent of precipitation.

Table 3. *Composition of precipitates obtained with iodinated rabbit serum proteins and rabbit antisera to iodinated horse serum globulins*

(The test antigen (5 ml. of a 0.0043-0.036% solution of iodinated rabbit serum proteins) was mixed with antiserum (4 ml.) and the mixture kept for 3.5 hr. at 37° and then overnight in the refrigerator. The centrifuged precipitates were washed twice with ice-cold 0.9% NaCl solution (0.5 ml. each time) and dissolved in water with the aid of a few drops of 0.5N-NaOH. The solutions were made up to 15 ml. and duplicate N and I determinations were made on 1 and 6 ml. samples respectively.)

Tube no.	Antigen added		Tests† on supernatant solution		Composition of precipitate				
	N* (μg.)	I* (μg.)	Antigen present	Antibody present	Total N (μg.)	Total I (μg.)	Antigen N (μg.)	Antibody N (μg.)	Antibody N/antigen N
1	74.8	67.3	+	0	178	19.2	21.4	157	7.3
2	37.4	33.6	+	0	213	19.6	21.8	191	8.8
3	29.9	26.9	±	0	184	16.1	17.9	166	9.3
4	18.7	16.8	tr.	f.tr.	152	10.6	11.8	140	11.9
5	14.9	13.4	f.tr.	f.tr.	105	5.5	6.1	99	16.2
6	9.0	8.1	f.tr.	±	32.6	1.4	1.5	31	20.7

* All the N and I values in this table are expressed as μg./ml. of antiserum used.

† Tested by the addition of antiserum and antigen to separate samples; 0, none detectable; f.tr., faint trace; tr., trace; ±, moderate and +, strong, indicate the extent of precipitation.

Table 4. *Composition of lipovitellin-antilipovitellin precipitates*

(Antigen: lipovitellin (containing ³²P); antibodies: the globulin fraction (labelled with ¹³¹I) of an antilipovitellin serum. All the weights given in this table are in terms of μg./ml. of antiserum used. Experimental details are given in the text. L=lipid (phospholipin); V=vitellin; AB=antibody.)

Antigen		Composition of the precipitate					
Lipid P (μg.)	Vitellin P (μg.)	Lipid P (μg.)	Vitellin P (μg.)	Antibody N (μg.)	Mol. ratios*		
					(L/V)†	(AB/V)	(L/AB)
77.5	48	26.7	5.8	217	136	1.30	104
103	64	30.0	6.9	253	129	1.27	102
129	80	37.2	7.6	270	147	1.24	119
155	96	40.0	7.6	257	155	1.16	134

* Assuming a molecular weight of 778 for phospholipin (palmito-oleo-lecithin; 3.99% P), and an arbitrary value of 100,000 for vitellin (0.92% P).

† The corresponding L/V ratio for the lipovitellin used in this experiment was 48.

P plus antibody I), and (c) the extracted precipitate after it had been de-iodinated (vitellin P).

Samples (6 ml.) of a solution of the iodinated globulins of an antiserum to lipovitellin were each treated with NaCl (0.6 g.) and a solution of lipovitellin (5 ml. of the stock solution suitably diluted with 10% NaCl). After being kept for 3 hr. at 37° and then overnight at about 4°, the mixtures were centrifuged and the supernatant solutions removed. The inside walls of the tubes were washed with 10% NaCl (5 drops) and the tubes were again centrifuged. Each precipitate was then washed three times with ethanol-ether (7:3 by vol.; 4 ml. each time). The extract was diluted to 15 ml. and the radioactivity of the solution was determined (using a liquid counter) and compared with that of the phospholipins of the original antigen. The lipid-free precipitates were treated with water (2 ml.) and 0.5N-NaOH (12 drops); they were left overnight at 37° and subsequently heated for 2-3 hr. in a boiling-water bath to complete the solution of the precipitates. Each solution was then diluted to 5 ml. and samples used for radioactivity determinations before and after de-iodination. Control experiments were made to determine how much lipovitellin was carried down

by an unrelated antigen-antibody precipitate (mustard gas sulphone-treated ovalbumin and its antiserum) and similar tests were made with the labelled antilipovitellin. The appropriate small allowances were made for this non-specific adsorption.

The results of this experiment are given in Table 4, which also includes some provisional calculated values for the relative numbers of molecules of lipid, vitellin and antibody present in each of the precipitates analysed. These molecular ratios should be regarded as having only relative significance where they involve vitellin molecules, since no information is available about the molecular weight of this insoluble protein.

DISCUSSION

Antigen-antibody precipitates can be analysed in various ways, depending largely on the nature of the antigen. Where the antigen contains no readily determinable 'unusual' element or group (e.g. iron

or dyestuff), the analysis largely depends on total N determinations. The method developed by Heidelberger and his colleagues (see Heidelberger & Kendall 1935; Heidelberger, 1939, 1946) and successfully used in the study of many different serological precipitates, requires, for systems containing an ordinary protein antigen, the following: (a) a determination of the total N content of the precipitate, and (b) tests to show that all the antigen has been precipitated (equivalence and antibody excess zones), or, for the antigen excess zone, a complicated analysis of the supernatant solution to determine how much antigen it contains.

With antigens and antibodies labelled with radioisotopes, the analysis of specific precipitates is comparatively simple; moreover, the method is equally applicable in the antigen excess zone. When a protein antigen is used and either the antigen or the antibody is labelled with a radio-isotope, the composition of the precipitate can be calculated from its radioactivity and total N content; if the isotope preparation is of high specific activity, the amount of precipitate required for the complete analysis is only very slightly greater than that needed for the total N determination. When both antigen and antibody are labelled, with different radio-isotopes which can be separately determined in mixtures containing both, the complete analysis can often be made on extremely small amounts of precipitate. Usually, however, there is no need for this strict economy of antigen or antibody, and in none of the experiments described here was an attempt made to work with minute amounts of material.

As antigens we have used ovalbumin, serum globulins and the mixed serum proteins containing ^{131}I , and biologically prepared ^{32}P -containing lipovitellin; in other experiments, not described here, protein antigens have been labelled by treatment with mustard gas sulphone (di-2-chloroethyl sulphone) containing ^{35}S . Both ^{131}I and ^{35}S are suitable labels for immunological studies, and, with radioactive material of high specific activity, iodine atoms or 'sulphone groups' can be introduced in such small amounts that there is no significant change in the immunological specificity of the antigen; alternatively, many of these groups may be introduced into the protein molecule and a labelled protein of new specificity obtained.

Our experiments with biologically prepared isotopically labelled protein antigens have been confined so far to those with lipovitellin and vitellin. The presence of the label in both the protein and phospholipin of ^{32}P -containing lipovitellin sometimes leads to complications, and moreover lipovitellin is a complex which may vary significantly from one preparation to another. Precise chemical information about this complex is rather scanty, but recently Mecham & Olcott (1949) have described the

isolation of a new water-soluble egg yolk phosphoprotein, which they named 'phosvitin' and which may account for 60–79% of the phosphoprotein P of egg yolk; a typical 'phosvitin' preparation had a P/N ratio of 0.815, compared with 0.14–0.17 for our lipovitellin preparations. Further investigations on the immunological properties of vitellin and lipovitellin appear desirable in the light of these observations.

Lipovitellin, although useful for the study of antibodies against lipids (Francis & Wormall, 1950), is not an ideal antigen for quantitative studies on specific precipitates. For experiments of this type, particularly for the study of serological reactions on a molecular basis, homogeneous and, if possible, crystalline protein antigens should be used. We have, therefore, used crystalline ovalbumin for many of our quantitative investigations. For other experiments, however, we have used the serum globulins and very occasionally mixed serum proteins, since these are sometimes more suitable than are proteins which are more 'foreign' to the animal body. For example, when chemically altered protein antigens are prepared from serum globulins, particularly those from the same species of animal used for the immunization, the parts of the antigen molecule involved in the precipitin reaction are largely, if not entirely, those containing the introduced group. Also, the zone of maximum precipitation is usually broader when unaltered or chemically altered mixed globulins, rather than crystalline proteins, are used as test antigens.

For the labelling of antibodies, we have found ^{131}I very satisfactory, and up to 1% of iodine can be introduced into the antibodies studied without serious loss of precipitating power. Much less is sufficient for tracer purposes, but we usually introduced 0.7–1.0% into the antibody molecule, equivalent to an average of 4.5–6.5 mol. of iodine (mainly or entirely in 3:5-diiodotyrosine groups) per mol. of antibody. With this degree of iodination it is highly probable that practically all the antibody molecules contain at least one tracer group (cf. Butement, 1948). If much less iodine is introduced the possibility of preferential precipitation of unlabelled, relative to labelled, antibody molecules cannot be excluded. No proof is yet available that the percentage of iodine in the antibody molecules in the precipitate is the same as that in the original iodinated antibodies, but this is probably true when most of the antibody is precipitated; whether it is true in the antibody excess zone we intend to study later. Pressman & Keighley (1948), Pressman (1949) and Butement (1948) have also used ^{131}I for labelling antibodies, and they have introduced amounts of iodine similar to those used here.

The molecular composition of specific precipitates has usually been studied with homologous systems,

e.g. ovalbumin-antiovalbumin, and there are relatively few quantitative observations on heterologous systems, particularly with chemically altered protein antigens. Our results are generally similar to those recorded for the ovalbumin and other homologous systems, but the zone of maximum precipitation for the heterologous system appears to be somewhat broader. Moreover, since the heterologous antigen is not perfectly 'adapted' to the antibodies, it is not completely precipitated except possibly in the region of extreme antibody excess (Figs. 1 and 2). The ratios of antibody to antigen in the precipitates are of the same order with heterologous and homologous systems (Fig. 3, and cf. Boyd, 1947, p. 243, and Landsteiner, 1945). None of the serological precipitates we have analysed contained fewer antibody than antigen molecules.

The lipovitellin-antilipovitellin precipitates contained considerably more phospholipin in relation to vitellin than did the lipovitellin used as antigen. Whatever arbitrary value is used for the molecular weight of the lipid-free protein vitellin, the molecular ratio of phospholipin to vitellin in the precipitates was about three times that for the lipovitellin (Table 4). These results provide further evidence of the capacity of rabbit antilipovitellin sera to precipitate phospholipins (Francis & Wormald, 1950).

SUMMARY

1. Quantitative studies on the precipitin reaction have been made with antigens and antibodies labelled with radioactive isotopes. This technique greatly simplifies the study of the composition of serological precipitates particularly in the antigen excess zone.

2. The results are given for analyses of serological precipitates containing antigens labelled with ^{32}P (lipovitellin) or ^{131}I (iodinated globulins and ovalbumin), and antibodies labelled with ^{131}I .

3. Up to 1% of iodine can be introduced into the proteins of many precipitating antisera without serious loss of their precipitating power.

4. Lipovitellin-antilipovitellin precipitates contained 100 or more molecules of phospholipin per molecule of antibody. The ratio lipid P: vitellin P in these precipitates was 4.3-5.3:1, compared with 1.6:1 for the sample of lipovitellin used. These results support the view previously put forward (Francis & Wormald, 1950) that antilipovitellin sera contain antibodies capable of precipitating phospholipins.

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REFERENCES

- Banks, T. E. (1946). *Brit. J. Radiol.* **19**, 333.
 Banks, T. E., Boursnell, J. C., Dewey, H. M., Francis, G. E., Tupper, R. & Wormald, A. (1948). *Biochem. J.* **43**, 518.
 Banks, T. E., Francis, G. E., Mulligan, W. & Wormald, A. (1949). *1st Int. Congr. Biochem. Abstr.* p. 476.
 Banks, T. E., Francis, G. E., Mulligan, W. & Wormald, A. (1950). *Nature, Lond.*, **165**, 111.
 Blum, F. & Strauss, E. (1923). *Hoppe-Seyl. Z.* **127**, 199.
 Boursnell, J. C., Dewey, H. M., Francis, G. E. & Wormald, A. (1947). *Nature, Lond.*, **160**, 339.
 Boyd, W. C. (1947). *Fundamentals of Immunology*, 2nd ed. New York: Interscience Publishers.
 Butement, F. D. S. (1948). *Nature, Lond.*, **162**, 731.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Francis, G. E. & Wormald, A. (1948). *Biochem. J.* **42**, 469.
 Francis, G. E. & Wormald, A. (1950). *Biochem. J.* **47**, 380.
 Greaves, R. I. N. (1946). The Preservation of Proteins by Drying. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 258.
 Heidelberger, M. (1939). *Bact. Rev.* **3**, 49.
 Heidelberger, M. (1946). 'Immunochemistry'. Chapter 29 in *Currents in Biochemical Research*, ed. by Green, D. E. New York: Interscience Publishers.
 Heidelberger, M. & Kendall, F. E. (1932). *J. exp. Med.* **55**, 555.
 Heidelberger, M. & Kendall, F. E. (1935). *J. exp. Med.* **62**, 697.
 Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immunology*. Springfield, Illinois: Charles C. Thomas.
 King, E. J. (1932). *Biochem. J.* **26**, 292.
 Landsteiner, K. (1945). *The Specificity of Serological Reactions*, p. 244, revised ed. Cambridge, Mass.: Harvard University Press.
 Markham, R. (1942). *Biochem. J.* **36**, 790.
 Marrack, J. R. (1938). The Chemistry of Antigens and Antibodies. *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 230.
 Mecham, D. K. & Olcott, H. S. (1949). *J. Amer. chem. Soc.* **71**, 3670.
 Perlmann, I., Chaikoff, I. L. & Morton, M. E. (1941). *J. biol. Chem.* **139**, 433.
 Pressman, D. (1949). *J. Immunol.* **63**, 375.
 Pressman, D. & Keighley, G. (1948). *J. Immunol.* **59**, 141.
 Proom, H. (1943). *J. Path. Bact.* **55**, 419.
 Shahrokh, B. K. (1943). *J. biol. Chem.* **147**, 109.
 Svedberg, T. & Eriksson-Quensel, I. B. (1935-6). *Tabul. biol., Berl.*, V, (4), 352.
 Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.
 Wormald, A. (1930). *J. exp. Med.* **51**, 295.
 Wormald, A. (1948). *Brit. med. Bull.* **5**, 333.