ON THE DIFFERENTIATION OF PROTEINS OF CLOSELY RELATED SPECIES BY THE PRECIPITIN REACTION.

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THE ultimate problem underlying many applications of the precipitin test, whether it be the determination of biological relationships, the identification of blood stains and other animal traces, or the detection of adulteration in food, is the recognition of the homologous protein (antigen) and its separation from closely allied heterologous proteins. Recognising that the antiserum is the main source of the precipitate in a precipitin reaction and having regard to the exact quantitative relations of antiserum, antigen and precipitate we have been able to arrange methods for the differentiation of proteins of closely related species and, we believe, to render more accurate the diagnosis of the source of individual proteins. To take a crucial instance, by means of an antiserum prepared with hen egg-white we have been able clearly to distinguish solutions of hen egg-white from all other avian egg-whites tested, including those of the duck, quail, partridge, pheasant and ostrich. So far as we know, Nuttall and Graham Smith alone have previously been successful in differentiating homologous and heterologous avian egg-albumens, and their methods appear to be more cumbersome than ours. Incidentally we have found that our results are not only consistent *inter se* but consistent also with the interpretation of the precipitin reaction which our previous observations had led us to adopt.

Historical. In 1900 Myers[1] and, a few months later, Uhlenhuth[2] demonstrated that antisera, prepared by injecting rabbits with hen egg-albumen, yielded precipitates when added to avian egg-albumens,

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the precipitation being more marked with homologous than with heterologous egg-albumens. In 1901 Uhlenhuth[3] found that a precipitin antiserum prepared with hen egg-white might cause precipitation in solutions of avian blood sera as well as in solutions of avian eggs. Further, by injecting a rabbit with goose egg-white, he obtained an antiserum which gave abundant and rapid precipitation with goose and duck eggs and well marked clouding with hen, guinea-fowl and pigeon eggs. He was led to conclude that it was not possible to distinguish the various kinds of eggs by the precipitin test, as he had done with different blood sera. In 1902 Gengou[4] stated that he could not observe any difference in the action of hen egg-white antiserum upon solutions of the egg-whites of the hen, duck, pigeon and turkey.

Nuttall, in a series of publications[5,6,7], confirmed and extended the original observations of Myers and Uhlenhuth, obtaining positive reactions with a hen-egg antiserum and a variety of avian and reptilian bloods, suggesting the "reptilian-avian" character of the reaction. He also introduced a "quantitative method for the measurement of the degree of the reaction" by estimating the bulk of the precipitate from measured quantities of the interacting dilutions.

In 1904 Nuttall[8] recorded two hen-egg antisera and one emu-egg antiserum, each of which gave the largest reaction only with the corresponding homologous protein and lesser reactions with other avian eggs and with some avian and reptilian blood sera. At Nuttall's suggestion Graham-Smith[9] extended his work with the result that their qualitative methods proved to be inadequate to distinguish the homologous from heterologous egg-whites, but Nuttall's quantitative method was successful in every case in which it was tested.

In an earlier paper[10] we summarised other methods that had been proposed for the differentiation of closely allied proteins by Nuttall, by Linessier and Lemoine, by Ewing, by Weichardt and by Uhlenhuth. Ewing[11], following a suggestion made by Uhlenhuth and others, tried the effect of progressively diluting the antiserum while maintaining the blood dilutions constant. He found that when added to various bloods in solutions of equal strength an antihuman serum in its highest dilution acted only upon human blood dilutions, and his other results were concordant.

In the same paper[10] we published a preliminary account of a method which we had independently devised, and which, though superficially similar, is fundamentally different from that of Ewing. To a series of

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fixed quantities of each protein to be tested there were added progressively diminishing amounts of the antiserum. The quantities of protein and of antiserum were regulated by the consideration that the quantity of protein, when homologous, should be sufficient and not much more than sufficient, to give a maximum precipitate with the greatest amount of antiserum employed.

Our method was based on the experimental finding that the precipitable substance is contained in the antiserum [10, 12, 13] and that there is a quantitative relation between the amount of precipitate and the amount of antiserum, provided the homologous protein is sufficient. Since then we have obtained experimental evidence [14, 15] that the antigen being in sufficient amount the weight of precipitate is proportional to the weight of antiserum engaged in the interaction. This last observation places the method on a scientific basis and offers some guarantee of its accuracy.

Experimental. We start from the experimental result that in certain conditions a given quantity of antiserum yields a definite weight of precipitate, provided that a sufficient amount of homologous protein be present. If the protein of the homologous species be replaced by the protein of any heterologous species, however closely related (as tested by the biological method), the weight of precipitate from that quantity of antiserum is diminished. It is not, however, generally practicable to weigh the precipitate from a given quantity of antiserum interacting with a quantity of unknown protein as a means of differentiation of proteins. But the same principle, adapted to other circumstances, may be employed to distinguish between closely related proteins. As an example we shall quote experiments which record the interactions between antisera for hen egg-white and the egg-whites of the hen, duck, quail, partridge, pheasant and ostrich, and by which the heterologous egg-whites of the different eggs were clearly distinguished from hen egg-white.

The antiserum, derived from a rabbit which had received six injections of hen egg-white (altogether equivalent to 6·27 gm. dried egg-white), was dried in vacuo over calcium chloride at 37° C. At the time the experiments were performed the antiserum had been dried for over two months. In the first experiment diminishing amounts of the antiserum were allowed to interact with constant quantities of the homologous and heterologous proteins. The antiserum solution was prepared by dissolving 0·13 gm. dried antiserum in 5·2 c.c. saline solution, so that 0·4 c.c. of the solution contained 0·01 gm. dried

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antiserum, 0·2 c.c. solution contained 0·005 gm. antiserum, and so on. Solutions of the various egg-whites were obtained by diluting 1 c.c. of egg-white from each of six kinds of eggs (hen, duck, quail, partridge, pheasant and ostrich) with 99 c.c. saline solution; and 0·1 c.c. of the solution of each kind of egg-white was placed in each of six tubes, so that six series of tubes were arranged, each series consisting of six tubes. The original antiserum solution was measured out in quantities equal to six times that required for each tube, and saline solution added in such quantity that the amount of diluted antiserum for six tubes measured 3 c.c. in all. Of this secondary dilution of antiserum 0·5 c.c. was transferred to each tube. In this way it was possible to measure the small amounts of antiserum with some approach to accuracy. The quantities of the interacting bodies in each series of tubes are given in Table I.

TABLE I.

No. of tube in each series	Weight of dried antiserum	Amount of the original solution of antiserum	Amount of saline solution added to original solution of antiserum	Amount of diluted egg-white (hen, duck, quail, partridge, pheasant and ostrich)
1	0·01 gm.	0·4 c.c.	0·1 c.c.	0·1 c.c.
2	0·005	0·2	0·3	0·1
3	0·002	0·08	0·42	0·1
4	0·001	0·04	0·46	0·1
5	0·0005	0·02	0·48	0·1
6	None	None	0·5	0·1

In conducting the experiments, precautions were taken against bacterial contamination. The reactions were allowed to take place at room temperature (about 18° C.) and the precipitates were read after 48 hours, as given in Table II.

TABLE II.

No. of tube in each series	Weight of dried antiserum	Precipitate with hen egg-white	Precipitate with duck egg-white	Precipitate with quail egg-white	Precipitate with partridge egg-white	Precipitate with pheasant egg-white	Precipitate with ostrich egg-white
1	0·01 gm.	2·5 mm.	1·0 mm.	0·8 mm.	0·8 mm.	1·0 mm.	0·5 mm.
2	0·005	1·0	0·3	0·3	0·5	0·5	0·3
3	0·002	0·3	trace	trace	trace	trace	trace
4	0·001	trace	trace	none	none	trace	none
5	0·0005	trace	none	none	none	none	none
6	none	none	none	none	none	none	—

The results show that the precipitate with hen egg-white was much greater than the precipitate with any heterologous protein, and that the differentiation is easily made by testing in this way with diminishing

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quantities of antiserum. Although 43 tubes were employed (including controls), the amount of dried antiserum required was only 0·13 gm., equivalent to 1·3 c.c. fresh antiserum. The method is therefore economical of material.

An unknown protein solution could be made comparable with the 1 °/₀ protein solutions above employed by so adjusting the dilution that 0·1 c.c. should yield with trichloracetic acid a precipitate measuring between 1 mm. and 2 mm. in narrow tubes, as described by us [12]. Then 0·1 c.c. of the unknown protein solution would contain approximately 0·0001 gm. of dried protein; and the test could be carried out by comparing this solution with similar dilutions of the homologous protein, and of a closely allied heterologous protein.

Further experiment showed that, when the quantity of heterologous protein interacting with 0·01 gm. dried antiserum is increased to produce the maximum precipitate obtainable from that amount of antiserum, the amount of precipitate is less than the full precipitate yielded by the same amount of antiserum interacting with a sufficiency of the homologous protein. An illustrative experiment is given in Table III which records the result of an experiment similar to that quoted in Tables I and II but carried out with another hen-egg antiserum. At the end of every 48 hours the superfluids were removed to clean tubes and treated with a fresh amount (0·1 c.c.) of the corresponding solution of egg-white.

TABLE III.

No. of tube	Weight of dried hen-egg antiserum	Amount of diluted egg-white	Precipitate in 48 hours	Addiment to superfluid of diluted egg-white	Precipitate from superfluid in 48 hours
1	0·01 gm.	0·1 c.c. (hen)	2·5 mm.	0·1 c.c. (hen)	0·3 mm.
2	0·01	0·1 c.c. (duck)	0·5	0·1 c.c. (duck)	none
3	0·01	0·1 c.c. (ostrich)	0·5	0·1 c.c. (ostrich)	0·5 mm.

Further addiments of 0·1 c.c. of the respective egg-white solutions to the superfluids produced no further precipitation. The readings show that the combined precipitates obtained with any heterologous protein did not equal the combined precipitates given by the homologous protein.

Another method of differentiating closely allied proteins has been described by us [16]. This method depends on the inhibition of the formation of precipitate by heated antisera, and particularly on the phenomena of "crossed inhibition." It is not, however, so simple as that described above, as it involves a knowledge of the inhibitory powers

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of the antisera employed, and requires a detailed examination of each antiserum before use. The results obtained in our work on "crossed inhibition" led us to suggest that the precipitate given by hen-egg antiserum and ostrich or any egg-albumen other than hen egg-albumen might be regarded as similar to that produced by ostrich-egg antiserum and any egg-albumen other than ostrich egg-albumen. It could be assumed that this precipitate resulted from the general avian character or component of the proteins used in the immunisation, while the increased precipitate produced by hen egg-albumen and hen-egg antiserum, or by ostrich egg-albumen and ostrich-egg antiserum, could be assumed to be due to the specific hen or ostrich character or component of the material used for injection.

In this connection some observations made on the eggs used for the experiment quoted in Tables I and II may be noted. After 48 hours the reactions recorded in Table II were completed, and the precipitates were read. The superfluids of tubes No. 1 in each series were removed to clean tubes, and to these superfluids certain addiments of the solutions of egg-white were made. The solutions were those used in the original experiment. The observations are detailed in Table IV, where the first four columns are merely a rearrangement of certain data from Tables I and II.

TABLE IV.

No. of tube in Tables I and II	Weight of dried hen-egg antiserum	Amount and nature of the original 1 % solution of egg-white	Precipitate at 48 hours	Amount and nature of the 1 % solution of egg-white added to clear superfluid	Precipitate from superfluid at 48 hours
1 (hen series)	0·01 gm.	0·1 c.c. (hen)	2·5 mm.	0·1 c.c. (ostrich)	none
1 (duck series)	0·01	0·1 c.c. (duck)	1·0	0·1 c.c. (ostrich)	trace
1 (quail series)	0·01	0·1 c.c. (quail)	0·8	0·1 c.c. (partridge)	none
1 (partridge series)	0·01	0·1 c.c. (partridge)	0·8	0·1 c.c. (hen)	1·5 mm.
1 (pheasant series)	0·01	0·1 c.c. (pheasant)	1·0	0·1 c.c. (partridge)	0·5 mm.
1 (ostrich series)	0·01	0·1 c.c. (ostrich)	0·5	0·1 c.c. (duck)	0·5 mm.

In the interpretation of these results the observations noted in Table III must also be considered. There it is seen that one addiment of the heterologous protein solution is sometimes sufficient to neutralise the whole of the general avian precipitin present (cf. tube No. 2); whereas in other cases a single addiment of the heterologous protein solution does not suffice (cf. tube No. 3). On our interpretation of the precipitin reaction this is equivalent to saying that in some cases a single addiment of heterologous protein suffices to throw out of solution the whole of the general avian precipitable content of the antiserum;

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whereas in other cases the whole of the general avian precipitable substance is not so discharged.

Among the results of Table IV similar phenomena appear. In the superfluids of the duck and quail series the addition of a different heterologous protein failed to reveal a precipitate, probably because the general avian precipitable content (precipitin) had been completely discharged in the previous interaction; whereas in the superfluids of the pheasant and ostrich series the additional different heterologous protein revealed the presence of some general avian precipitable substance (precipitin) remaining undischarged after the primary interaction. In the superfluid of the hen series the addition of ostrich egg-white failed to yield a precipitate, probably because the primary interaction with the homologous protein had completely eliminated the general avian precipitable substance from the antiserum together with most of the specific hen "precipitin." In the superfluid of the partridge series the addiment of hen egg-white precipitated the specific anti-substance (precipitin) for hen egg-white, giving therefore a large precipitate, the primary interaction with a heterologous protein having affected only the general avian antisubstance.

These results have a further interest in the light of similar "saturation phenomena" that may be exhibited by haemolytic antisera.

In order not to load our paper with experimental detail we have quoted only a few of our observations in illustration of our points. But they are supported by many similar experiments which we have carried out at different times and always with concordant results.

CONCLUSIONS.

(1) It is possible clearly to distinguish heterologous proteins of closely related species from the homologous protein by precipitin interactions arranged with regard to the fact that in the conditions of the experiment the weight of precipitate is proportional to the weight of antiserum employed.

(2) By "saturation experiments" it is possible to indicate in an avian egg-white antiserum the presence of a general avian antisubstance (precipitin) together with the specific antisubstance.

(3) The consistency of these results with our interpretation of the precipitin reaction lends further support to the working hypothesis which we have advanced in previous papers.

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