On the Main Source of "Precipitable" Substance and on the Rôle *of the Homologous Proteid in Precipitin Reactions.*

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In suitable conditions outside the animal body the most obvious result of the interaction of a precipitin antiserum with its homologous proteid is the appearance of a flocculent precipitate in previously clear solutions. The accepted explanation is that the antiserum contains a precipitating substance or precipitin, through the agency of which the precipitable substance, represented by the homologous proteid, is thrown out of solution. It would generally be admitted that the precipitin to some extent enters into the constitution of the precipitate, since it is through combination with definite quantities of precipitin that the proteid is by most observers supposed to be rendered insoluble. Nevertheless, even if that view of the interaction be adopted, and still more if the precipitin be regarded as the analogue of a ferment or as a non-proteid body altogether, it has been generally taken for granted that the precipitate is constituted essentially by the precipitation of the test proteid, and this conception of the source of the "precipitable" substance has dominated all hypotheses and statements regarding the nature of precipitin reactions.

So far, however, as our observations go, we are led more and more definitely to the conclusion that the great bulk of the substance that is thrown out of solution and that goes to form the precipitate, is supplied by the antiserum and not by the test proteid, and that, whatever part in the interaction is played by the test proteid, it is not merely that of supplying a substance to be thrown out of solution by the precipitin antiserum.

That the antiserum itself is the main source of the "precipitable" substance is indicated by the fact that quantities of the test proteid many times too minute to yield an appreciable precipitum with ordinary proteid precipitants may still yield distinct precipitates on the addition of a suitable antiserum. Even if the whole of the test proteid were thrown out of solution, it would be altogether insufficient to account for the mass of the

precipitum obtainable in the latter instances. But it can be shown that the test proteid is not all removed from the solution even when minute quantities interact with antiserum to yield definite precipitates. On the contrary, when the clear superfluid is transferred to a fresh tube and a second dose of antiserum is added, there is deposited a second precipitate, and this process has been repeated six times, each fresh addition of antiserum resulting in a fresh precipitate. In these circumstances it is hard to conceive an adequate source of "precipitable" substance other than the antiserum. Supported by other facts also, this conception of a precipitable content in precipitin antisera is not only in harmony with the known phenomena of precipitin reactions, but appears to offer a more consistent explanation of these phenomena and of certain anomalies that arise on the assumption that the precipitable substance is derived wholly or mainly from the test proteid.

Our experiments are based on the exact measurement of the substances concerned. The interacting quantities were often very minute, and we have been aided in their measurement by the use of dried material. When it was more convenient to employ undried material, a known volume was dried and weighed, so that our results might be expressed in terms of the weights of dried equivalents. We have independently determined the fact, recognised by the majority of observers, that careful desiccation of the substances concerned does not impair their efficiency.

At a room temperature averaging 18° C. and in the dilutions employed by us, we have repeatedly noted that a time interval of less than 48 hours may be insufficient for complete interaction, though in certain circumstances the reaction may be regarded as complete in 24 hours.

For such prolonged exposures, and more especially when the same solutions were tested again and again by repeated addition of proteid or of antiserum, it became imperative to exclude bacteria as effectively as possible. This we were able to do by collecting and storing our material aseptically, by sterilising all apparatus and saline solutions employed, by filtration through porcelain of suspected solutions, and generally by taking the usual precautions against bacterial contamination such as plugging the tubes with sterile cotton wool. The only stage at which contamination was likely to occur was that of shaking the tubes to ensure thorough mixing of the solutions. In the relatively few instances in which bacterial growths appeared we had no difficulty in distinguishing the sediment due to bacteria from the flocculent matter thrown out as a result of precipitin interactions. Nevertheless, when small precipitates were in question, we rejected contaminated tubes.

In reading the precipitates we have made a careful approximation to the length of the tube occupied by the deposit, a graduated scale being held

alongside. In any one experiment it is possible to compare and classify the precipitates with accuracy, and in different experiments or in different stages of the same experiment a reliable comparison between the deposits may be made by taking sufficient intervals between the readings such as might be represented by traces, 0·5 mm., 1 mm., 2 mm., and other multiples of 1 mm.

Ordinary test-tubes and samples of the small test-tubes commonly employed in precipitin reactions we found to be unsuited for the reading of the smaller precipitates. Consequently, we were obliged to make our own tubes from narrow glass tubing not exceeding 4 mm. in diameter, the melted ends being simply allowed to fall together in the flame. For special purposes tubing of about 5 mm. diameter was employed.

The following experiments afford a comparison between the precipitates obtainable from the interaction with precipitin antisera of varying quantities of proteid in solution and those obtainable from corresponding proteid solutions by addition of proteid precipitants.

In *Experiment I* two corresponding series of tubes were prepared containing from 0·05 to 0·00000005 gramme dried horse serum in 0·5 c.c. saline solution (0·75 per cent. sodium chloride), so that the dilution of dried horse serum in 0·5 c.c. saline varied from 1 in 10 to 1 in 10,000,000. A control tube containing 0·5 c.c. saline alone was also prepared. To each tube of the first series and to the control was added 0·01 gramme dried antiserum (prepared in a rabbit by injection of dried horse serum) previously dissolved in 0·5 c.c. saline solution. To each tube of the second series was added 0·5 c.c. of a 10-per-cent. solution of trichloracetic acid. The precipitates were read after 48 hours, as shown in Table I.

Table I.—Primary Interactions. Experiment I.

No. of tube.	Grammes of dried horse serum in each tube.	Dilutions of dried horse serum in 0·5 c.c. saline.	Precipitates in tubes to which 0·01 gramme dried antiserum was added.	Precipitates in tubes to which 0·5 c.c. of 10-per-cent. trichloracetic acid was added.
			mm.	mm.
1	0·05	1 in 10	1	60
2	0·005	1 „ 100	1·5	21
3	0·0005	1 „ 1,000	1·5	4
4	0·00005	1 „ 10,000	1·5	1·5
5	0·000005	1 „ 100,000	0·5	None
6	0·0000005	1 „ 1,000,000	0·3	None
7	0·00000005	1 „ 10,000,000	0·3	None
8	Control	Saline alone.	None.	—

Hence, in Tubes Nos. 5, 6, and 7 definite small precipitates are given by precipitin reactions in which the total amount of homologous proteid is

insufficient to yield any visible precipitate when acted on by trichloracetic acid. Salicyl sulphonic acid, hydroferrocyanic acid, picric acid, mercuric chloride, and heat also failed to demonstrate the presence of 0·000005 gramme dried serum in 0·5 c.c. saline. Similar observations have already been recorded, notably by Linossier and Lemoine, as indicating the delicacy of the precipitin test for minute traces of homologous proteid, but we are not aware that the inference that the antiserum must be the main source of the substance that goes to form the precipitate has previously been made.

It is also noteworthy that the maximum precipitate is given by an amount of homologous proteid which, as will next be shown, is adequate to neutralise all (or practically all) the precipitin, and that no further increase of the homologous proteid will augment that precipitate. In view of the phenomena that we have yet to describe, we should therefore regard a precipitate of about 1·5 mm. as representing the precipitable content of 0·01 gramme of this particular antiserum. And, roughly speaking, since a precipitate of 1·5 mm. is given by trichloracetic acid with 0·00005 serum, we may also say that the weight of "precipitable" substance in 0·01 gramme of this antiserum may be represented by a figure of the same order of magnitude as 0·00005 gramme, in other words, that one part of this antiserum contains 0·00x. part of "precipitable" substance.

If after 48 hours one-fifth part of the clear superfluid from each precipitin reaction be transferred to each of two clean tubes, A and B, and if one series, A, be tested for residual precipitin by the addition of 0·0001 gramme dried horse serum in 0·5 c.c. saline, while the other series, B, is searched for residual horse serum by addition of 0·01 gramme dried antiserum in 0·5 c.c. saline, the following secondary interactions occur, the precipitates being read in 48 hours, as shown in Table II :—

Table II.—Secondary Interactions. Experiment I.

No.	Precipitates in A series (+ horse serum).	Precipitates in B series (+ antiserum).
	mm.	mm.
1	Not tested	Not tested.
2	None	2·5
3	None	1·5
4	0·3	0·5
5	0·5	0·3
6	0·5	0·3
7	0·5	0·3
8	0·5	None

These results show that the so-called precipitin was completely neutralised in Tubes Nos. 2 and 3, partially neutralised in Tube No. 4, and not

noticeably diminished in Tubes Nos. 5, 6, and 7, when the precipita are compared with that given in the control Tube No. 8. The homologous proteid, on the contrary, was not exhausted in any tube, and in spite of the facts that only one-fifth of the amount originally present was taken, and that it had already reacted with antiserum to yield definite precipita, it was still capable of eliciting from a second supply of 0·01 gramme antiserum precipitates not less than those obtained in the primary interactions. An apparent exception is Tube No. 4, in which occurred a deposit much smaller than that primarily given. But the smaller precipitate in this instance reinforces our argument, since it is in all probability attributable not to exhaustion of the homologous proteid, but to the fact that the amount of horse serum (0·00005 gramme) originally present was just insufficient to neutralise 0·01 gramme antiserum, and that the amount actually present in the secondary reaction was 0·00001 gramme (already once acted on), making the interacting quantities more nearly comparable to those in the primary Tube No. 5.

In Tubes Nos. 4, 5, 6, and 7 uncombined precipitin coexisted with uncombined homologous proteid in the clear superfluids, an observation repeatedly made by Linossier and Lemoine, Eisenberg, Ascoli, and others.

Assuming for the moment that our two main conclusions were correct, (1) that the precipitum is derived mainly from the antiserum, and (2) that the homologous proteid is relatively not exhausted in any interaction, we were naturally led to investigate the effect of adding to minute quantities of the homologous proteid amounts of precipitin antiserum larger than those used in Experiment I. The results were confirmatory of our hypotheses.

Experiment II.—Taking an amount of dried horse serum (0·000005 gramme), sufficiently minute to escape detection by trichloracetic acid and other proteid precipitants (*cf.* Tube No. 5, Experiment I), we allowed it to interact with increasing amounts (0·01 gramme, 0·05 gramme, 0·1 gramme) of dried horse antiserum, the salt solution in each tube being made up to 1·1 c.c. The results are summarised in Table III.

Table III.—Experiment II.

No.	Amount of dried horse serum in grammes.	Amount of dried antiserum in grammes.	Precipita in tubes to which antiserum was added.	Precipita obtainable by ordinary proteid precipitants.
			mm.	
1	0·000005	0·01	0·3	Inappreciable
2	0·000005	0·05	1	„
3	0·000005	0·1	2	„

These results indicate that by increasing the amount of precipitin antiserum there may be obtained precipitates that are enormous relatively to those obtainable from the total amount of homologous proteid in solution. In view of these observations it becomes still more difficult to explain the origin of the precipitate otherwise than from the antiserum.

When similar observations are carried out with antisera for egg albumins, the results are in accordance with those obtained for horse serum and antiserum.

Experiment III.—Two corresponding series of tubes were prepared, as in Experiment I, containing from 0·0005 to 0·00000005 gramme dried hen egg white in 0·5 c.c. salt solution. To each tube of the first series and to a control was added 0·01 gramme dried antiserum (prepared in a rabbit by injection of dried egg white) previously dissolved in 0·5 c.c. salt solution. To each tube of the second series was added 0·5 c.c. of 10-per-cent. trichloracetic acid. The precipitates were read after 48 hours, as shown in Table IV.

Table IV.—Primary Interactions. Experiment III.

No.	Amount of dried egg white in grammes.	Dilutions of dried egg white in 0·5 c.c. saline.	Precipita in tubes to which 0·01 gramme dried antiserum was was added.	Precipita in tubes to which 0·5 c.c. 10-per-cent. trichloracetic acid was added.
			mm.	mm.
1	0·0005	1 in 1,000	1·5	5
2	0·00005	1 ,, 10,000	1·5	1·5
3	0·000005	1 ,, 100,000	1	Minute trace
4	0·0000005	1 ,, 1,000,000	0·3	None
5	0·00000005	1 ,, 10,000,000	0·3	None
6	Control	Saline alone	None	—

Again it appears that definite small precipita are obtainable by the interaction with precipitin antiserum of amounts of homologous proteid inadequate to yield a trace of deposit when thrown out of solution by trichloracetic acid.

After 48 hours one-fifth part of the clear superfluid from each precipitin reaction was transferred to each of two clean tubes, A and B. Series A was tested for residual precipitin by the addition of 0·1 c.c. of 1-per-cent. fresh egg white in salt solution (equivalent to 0·00012 gramme dried) to each tube, while in series B the presence of homologous proteid in excess was revealed by additions of 0·01 gramme dried antiserum in 0·1 c.c. saline. The total fluid in each tube was made up to 0·6 c.c. by addition of salt solution. The precipitates in these secondary reactions were read in 48 hours, as in Table V.

Table V.—Secondary Interactions. Experiment III.

No.	Precipita in A series (+ egg white).	Precipita in B series (+ antiserum).
	mm.	mm.
1	0	2
2	0	2
3	0·5	0·5
4	1	0·3
5	1	0·3
6	1	None

Among the data afforded by this experiment may be indicated (1) the complete neutralisation of the precipitin in the first two tubes only, and the coincidence of this point with the maximum precipitate obtainable in the primary interactions from 0·01 gramme of this antiserum; (2) the inappreciable exhaustion of the homologous proteid as a result of the primary interactions; and (3) the coexistence of uncombined egg white and its antiserum in the clear superfluids after the primary interactions are completed.

When larger amounts of egg antiserum were employed, correspondingly larger precipita were obtained, the masses of which were still more strikingly incompatible with an origin from the homologous proteid available.

Experiment IV.—To two tubes, each containing 1 c.c. of fluid antiserum (approximately equivalent to 0·1 gramme dried) prepared by injection of fluid egg white, there was added 0·1 c.c. of 10-per-cent. fresh egg white in salt solution (equivalent to 0·0012 gramme dried) and 0·1 c.c. of 1-per-cent. fresh egg white in saline (0·00012 gramme dried) respectively. The precipitates were read in 48 hours, with the results shown in Table VI.

Table VI.—Primary Interactions. Experiment IV.

No.	Amount of egg white in grammes.	Amount of antiserum in grammes.	Precipita in 48 hours.
			mm.
1	0·0012	0·1	7 (compact)
2	0·00012	0·1	11 (looser)

The precipitate in Tube No. 2 decreased to 9 mm. on the third day, and, though kept under observation up to the seventh day, it did not shrink below 8·5 mm. The same amount of egg albumin (0·00012 gramme dried) when precipitated by trichloracetic acid would yield a deposit not exceeding

2·5 mm. Hence the precipitum resulting from the precipitin reaction, notwithstanding its looser consistence, cannot be explained as a derivative of the homologous proteid alone.

After 48 hours 0·1 c.c. of the clear superfluid from each tube was transferred to each of two clean tubes, A and B. To each A tube was added 0·00012 gramme egg white in 0·5 c.c. saline; to each B tube 0·01 gramme antiserum in 0·5 c.c. saline. The precipitates in 48 hours are given in Table VII.

Table VII.—Secondary Interactions. Experiment IV.

No.	Precipita in A series (+ egg white).	Precipita in B series (+ antiserum).
	mm.	mm.
1	0·5	1·5
2	1·5	0·5

Therefore, both precipitin and homologous proteid coexisted uncombined in the clear superfluids; the precipitin was largely neutralised in the first tube, but not much in the second; the homologous proteid was not noticeably exhausted in any tube. The last result is the most important for our present purpose.

Tertiary Interactions.—Having noted that the secondary interactions invariably revealed the presence of homologous proteid not noticeably exhausted, whether the proteid were of the nature of egg white or of the nature of serum, we submitted some of the superfluids of the secondary interactions to the influence of a fresh supply of proteid and of antiserum. The addition of proteid revealed the presence or absence of precipitin according as it had not or had previously been neutralised. The addition of antiserum invariably revealed the presence of unexhausted homologous proteid by the deposition of precipitates which were not noticeably smaller than if the same quantities had interacted originally. It is unnecessary to detail these observations, since similar results are more strikingly displayed in the following experiments:—

Exhaustion Experiments.—These observations were so unexpected and so much at variance with accepted views regarding the precipitation of the homologous proteid that we instituted a series of experiments to try if, by successive additions of antiserum to the clear superfluids remaining after each interaction, we could exhaust the homologous proteid. As the following experiments show, we are not in a position to say whether the proteid is or

is not appreciably used up as a result of interaction with precipitin antisera. Yet we have no conclusive evidence that it is so used up, and we have definite evidence that it is not entirely removed by any number of interactions or amounts of precipitin antiserum with which it has been tested.

Experiment V. First Interaction.—To 0·5 c.c. of 1-per-cent. fresh egg white in salt solution (equivalent to 0·0006 gramme dried) there was added 4·5 c.c. salt solution, and then 0·01 gramme dried antiserum (prepared in a rabbit by repeated injections of fluid egg white) in 0·2 c.c. saline. After 48 hours there was a deposit measuring between 0·5 mm. and 1 mm., and some flocculi in suspension.

Second Interaction.—The superfluid was then filtered, and to 4 c.c. of the filtrate was again added 0·01 gramme antiserum in 0·2 c.c. saline. In 48 hours the precipitate again exceeded 0·5 mm., and there was also some suspended matter.

Third Interaction.—The process was repeated, and 3 c.c. of filtrate plus 0·01 gramme antiserum yielded a precipitate about 1 mm. in 48 hours.

Fourth Interaction.—2·5 c.c. filtrate plus 0·01 gramme antiserum gave in 48 hours a large precipitate of 2·5 mm.

Fifth Interaction.—1·5 c.c. filtrate plus 0·01 gramme antiserum gave in 72 hours a 2-mm. precipitate.

Sixth Interaction.—The filtrate, measuring 1 c.c., was now divided equally between two tubes, A and B. To tube A was added 0·1 c.c. of 1-per-cent. fresh egg white in salt solution (0·00012 gramme dried), to tube B 0·01 gramme antiserum as before. After 48 hours the precipitates read in tube A 0·5 mm., in tube B 2 mm.

The first four interactions took place in tubes of about 5 mm. diameter, the others in tubes of 4 mm. diameter.

The smaller precipitates in the earlier interactions were probably due partly to the longer column of fluid through which they had to settle, and partly to the greater dilution of the precipitin antiserum retarding the reaction and rendering it not quite complete even in 48 hours.

The fluids did not altogether escape bacterial contamination, but up to the final interaction there was never more than a faint cloud beneath the aërial surface of the fluid. There was never any surface scum or pellicle, or any bacterial clouding in the deeper parts of the tube. The precipitates were distinctly flocculent, and could not have been mistaken for bacterial deposits. These remarks also apply to the other exhaustion experiments to be later described.

Notwithstanding, therefore, the progressive diminution of the available homologous proteid at each stage and the successive additions of antiserum,

the result is to show that in the final tube A there was no great residue of precipitin, whereas in tube B there was still sufficient homologous proteid to give the maximum precipitate from 0·01 gramme of this antiserum. This amount of homologous proteid could not exceed one-tenth of that orginally present, that is, it could not exceed 0·00006 gramme, and had already been acted on five times. These results are summarised in Table VIII:—

Table VIII.—Experiment V.

No. of interaction.	Amount of egg white remaining at each stage.	Total amount of fluid at each stage.	Amount in grammes of antiserum added at each stage.	Duration of each interaction.	Precipita at end of each stage.
	gramme.	c.c.		hrs.	mm.
1	0·0006	5	0·01	48	0·5+
2	0 00048 (once acted on)	4	0·01	48	0·5+
3	0·00036 (twice acted on)	3	0·01	48	1
4	0·0003 (three times acted on)...	2·5	0·01	48	2·5
5	0·00018 (four times acted on) ...	1·5	0·01	72	2
6	0·00006 (five times acted on) ...	0·5	0·01	48	2

In *Experiment VI* a much smaller amount (equivalent to 0·000000012 gramme dried) of egg white was originally taken and was allowed to react with 0·01 gramme dried antiserum (prepared in a rabbit with dried egg white). At the end of 48 hours the precipitum was noted, the superfluid was filtered, and a fresh supply of 0·01 gramme antiserum added. This process was repeated again and again, as summarised in Table IX. The precipitates were distinctly flocculent:—

Table IX.—Experiment VI.

No. of inter-action.	Amount in grammes of egg-white remaining at each stage.	Amount of fluid at each stage.	Grammes of antiserum added at each stage.	Duration of each inter-action.	Precipita at end of each interaction.
		c.c.		hrs.	mm.
1	0·000000012	5	0·01	72	slight = 0·3
2	0·0000000096 (once acted on)	4	0·01	48	„ = 0·3
3	0·0000000072 (twice acted on)	3	0·01	48	„ = 0·3
4	0·0000000048 (three times acted on)	2	0·01	72	„ = 0·3
5	0·000000003 (four times acted on)	1·25	0·01	48	more abundant = 0·5
6	0·0000000018 (five times acted on)	0·75	0·01	48	„ = 1

We omitted to test the superfluid of the final interaction with a relatively large amount of egg white, but, doubtless, had we done so, a large residuum of uncombined precipitin would have been revealed. The important fact, however, remains, that the homologous proteid, so far from showing evidence of exhaustion, yields more abundant precipitates at the end than at the beginning of the series, possibly owing to the increasing concentration of the precipitin.

In the next experiment a similar process of repeated interaction with antiserum was applied concurrently to three tubes containing different amounts of the homologous proteid.

Experiment VII.—The original amounts of fluid egg white were equivalent in Tube No. 1 to 0·000006 gramme, in Tube No. 2 to 0·000012 gramme, in Tube No. 3 to 0·000024 gramme. Of dried antiserum (prepared by injection of fluid egg white) 0·01 gramme was added to each tube, and the same dose was repeated at each successive interaction. The amount of fluid in the original tubes was made up to 1·1 c.c. with salt solution. At each stage the clear superfluids were transferred by means of a pipette to clean tubes. The amounts were not accurately measured, but about 0·2 c.c. of the fluid was left behind each time. The tubes were 4 mm. in diameter. The results are summarised in Table X:—

Table X.—Experiment VII.

No. of interaction.	Tube No.	Duration of each interaction.	Precipita at end of each stage.
		hrs.	mm.
1	1	48	1
	2	24	1
	3	24	1
2	1	48	1·5
	2	24	1·5
	3	24	1·5
3	1	48	1·5
	2	72	1·5
	3	72	1·5
4	1	48	0·3
	2	48	0·3
	3	48	0·5

At the conclusion of the fourth interaction the clear superfluids were removed as before, and an amount of fresh egg white (equivalent to 0·00024 gramme dried) was added to each. Within 48 hours a large precipitate between 2·5 and 3 mm. appeared in each tube, indicating a considerable excess of uncombined precipitin.

This experiment is somewhat unsatisfactory, for two reasons—the time intervals were not adjusted to ensure completion of each interaction, and the amount of superfluid removed at each stage was not accurately determined. We would not have recorded it at all, were it not for the fact that it is the only indication we have met that the homologous proteid may be diminished as a result of interaction with its precipitin. Even so it is not until after the third interaction that any diminution of homologous proteid is indicated, and we are unable to decide whether that is due to the proteid having been used up to any appreciable extent, or to its amount having been so reduced by successive subdivisions that it is no longer sufficient to produce the full precipitate from 0·01 gramme of this antiserum.

The cumulative evidence of these and other experiments, all of which give concordant results, renders it increasingly difficult to trace the precipitum to an origin other than the antiserum itself. Presumably the "precipitable" substance in the antiserum is the body commonly known as the precipitin. In any interaction, however, it is the homologous proteid that in some way renders the precipitin insoluble, and this it does without sensibly diminishing its capacity for further effective interaction with fresh precipitin.

Comparison of Antisera Prepared in the Rabbit with Natural Rabbit Serum.

Being led by our observations to the conclusion that an antiserum, prepared in a rabbit by repeated injections of some alien proteid, contained a substance or substances in sufficient abundance to yield definite precipita when thrown out of solution by interaction with the homologous proteid, which substance or substances the natural serum of the rabbit did not contain, we began a series of experiments to determine whether or not it were possible to obtain evidence of other differences between antisera and natural sera from the rabbit besides those revealed by precipitin tests.

Table XI.

Weight in grammes of 5 c.c. of natural rabbit sera (when dried).	Average weight.	Weight in grammes of 5 c.c. of antisera prepared in rabbit (when dried).	Average weight.
	gramme.		gramme.
0·4937		0·5273 (ox serum)	
0·4681		0·5174 (horse serum)	
0·4471	0·446	0·5118 (ostrich egg I)	0·501
0·4228		0·4980 (ostrich egg II)	
0·3998		0·4878 (hen egg I)	
		0·4675 (hen egg II)	

In the first place equal volumes of various antisera prepared in the rabbit and of the natural serum of several rabbits were dried to constant weight over calcium chloride at 37° C. *in vacuo*, and weighed. In several instances the mean of two concordant estimations was taken. The results are shown in Table XI.

The difference between the mean weight of antiserum and that of natural serum becomes all the more striking when it is borne in mind that most of the rabbits had lost weight during the process of immunisation, whereas the control rabbits were all in good condition.

We next examined by fractional heat coagulation two hen egg antisera prepared in the rabbit, and compared them under the same conditions with natural rabbit serum, with hen egg white, and with one of the above hen egg antisera after interaction with hen egg white. In the last case 1·3 c.c. of fluid hen egg antiserum reacted for 72 hours with 0·3 c.c. of 1-per-cent. hen egg white in normal salt solution and yielded an abundant precipitate. The clear superfluid was then removed, and treated in the same way as the other substances investigated.

The fluid antisera, sera, and egg white were diluted 20 times with normal salt solution and acidified to an equal degree with acetic acid. Some preliminary experiments were necessary to determine the most suitable quantity of the latter.

The results are given in the following table (XII), in which the lower figure of each pair presents the temperature in degrees centigrade at which opalescence was observed, the higher that at which flocculation and complete precipitation occurred. Clear filtrates were obtained after each heating, and from 30 to 40 minutes were allowed at each temperature to remove the proteid.

Table XII.

Hen egg antiserum.	Neutralised antiserum.	Rabbit serum.	Egg white.
59—62	53—60	56—60 64—66	59—62
68—70 71—72	68—70	69—70	68—70
74—75	73—74	73—74	72—74
76—80	76—80	78—80	
84—86	84—86	82—84	

The figures indicate that in the antiserum there was present a body coagulating at 72° C., and disappearing after inaction with egg white. The precipitates that separated from the antiserum at this temperature and at

70° C. were dense flocculent masses, which settled to form large deposits. Such large deposits were not observed in the neutralised antiserum nor in natural serum at these temperatures. Further experiments on these lines are in progress.

The Precipitable Content and Precipitability of Antisera.

When a given amount (say 0·01 gramme) of antiserum is completely neutralised by its homologous proteid, so that no precipitin can be detected in the superfluid, then no further increase in the amount of homologous proteid will augment the precipitum, and that precipitum may be taken to represent the entire precipitable content of the given amount of that antiserum. Any reliable method of measuring the precipitum, such as that devised by Nuttall, would afford an indication of the precipitable content of an antiserum, provided that the amount of homologous proteid interacting were sufficient to neutralise all the precipitin within the limits of time allowed. As the complete neutralisation of the precipitin is not taken into consideration, and is not always secured by Nuttall's original method, we introduced a modification whereby complete neutralisation of the precipitin is secured by interaction with larger amounts of the homologous proteid. The bulk of the precipitate measured in this way affords a datum by means of which the precipitable content of different antisera may be compared.

Numerous observations serve to show that the precipitum is of a proteid nature, and, in discussing the primary interactions in Experiment I, we indicated another way in which the precipitable content may roughly be expressed—in terms of the amount of proteid to which the maximum precipitum from a given amount of antiserum is approximately equivalent. Thus we saw that the precipitable content of 0·01 gramme of the horse antiserum of Experiment I was $0{\cdot}0000x$ gramme. The precipitable content of the same amount of the egg antiserum of Experiment III might be similarly represented by a figure of the same order of magnitude.

Estimated by our modification of Nuttall's method, the precipitum from 0·01 gramme of the horse antiserum of Experiment I, and of the egg antiserum of Experiment III, measured in each instance 0·024 c.c. In the common acceptation of the term these antisera would rank as equally "powerful," that is, capable of throwing out of solution equal amounts of their respective homologous proteids. We should prefer to say that the precipitable content of the two antisera was equal.

Yet there is a striking difference between them, unrevealed by Nuttall's method or by our modification, for the amount of the homologous proteid

sufficient to neutralise all the precipitin in 0·01 gramme of antiserum is, roughly speaking, ten times greater in the case of the horse antiserum (0·0005 gramme, *cf.* Tube No. 3, Experiment I) than in the case of the egg antiserum (0·00005 gramme, *cf.* Tube No. 2, Experiment III). These data are not to be taken as accurately determining the minimum neutralising amounts of homologous proteid for 0·01 gramme of the antisera, as the intervals above and below the critical points are too great. Nevertheless they serve the purpose of indicating the nature of the difference between the two antisera in question. They show that the precipitable content of the egg antiserum is more easily discharged than that of the horse antiserum, since it requires less of the homologous proteid for complete neutralisation and precipitation.

In estimating the capacity of an antiserum for interaction with its homologous proteid, two factors, therefore, have to be considered: (1) the precipitable content of the antiserum, and (2) its precipitability. So far as our observations go, these factors appear to be mutually independent.

The results of operating with antisera whose precipitable content is not easily discharged are of interest, since they indicate how erroneous impressions may arise regarding the precipitation of the homologous proteid. In such cases the precipitates may show a progressive increase with each increase of the homologous proteid and only small precipitates with small amounts of the proteid.

In the readjustment of atom complexes that may take place within the animal body when its proteid molecules repeatedly encounter alien proteid molecules, as in processes of immunisation, it is not unreasonable to suppose that the reaction on the part of the host results in the formation of precipitable molecules which are endowed in certain circumstances with many, in other circumstances with few, atom complexes capable of interacting with corresponding atom complexes of the homologous proteid molecule. It is possible that the precipitable content of such an antiserum is determined by the number of precipitable molecules in a given molecule of the antiserum, while its precipitability is conditioned by the number of susceptible atom complexes that each molecule possesses.

The Rôle *of the Homologous Proteid in Precipitin Reactions.*

Our observations do not enable us to decide whether the homologous proteid is or is not diminished as a result of interaction with precipitin antisera. We have, however, definite evidence that it is not wholly exhausted and no conclusive evidence that it is sensibly exhausted even after

repeated interactions with 0·01 gramme or single interactions with 0·1 gramme of antiserum, however minute the original amount of proteid may have been.

That the interaction is of a chemical nature we have no reason to doubt. That a given amount of antiserum will remove from solution definite amounts of its homologous proteid we may accept on the understanding that the amounts of proteid so removed are exceedingly minute. But that is not what is usually meant when it is said that the homologous proteid combines quantitatively with the precipitin to form the precipitum. What is meant is that measurable amounts of both substances disappear from the superfluid after each interaction. That this is true of the substance contributed by the antiserum we have abundant evidence; that it is true of the homologous proteid we have no evidence whatever.

Consistently with our observations, therefore, the results of a precipitin interaction cannot be stated in the form of a simple chemical equation. We are not in a position to formulate any definite hypothesis, but we may at least infer that the processes underlying the interaction are of a more complex nature. From the fact that the homologous proteid is not appreciably diminished, it is suggested that the proteid may exert a catalytic effect on the precipitable substance of the antiserum, whereby a molecular rearrangement is induced and the substance is thrown out of solution.

Conclusions.

1. The homologous proteid is not wholly removed from the superfluid of a precipitin interaction, whether it is more than sufficient or less than sufficient to neutralise all the precipitin present.

2. Conclusive evidence that the homologous proteid is sensibly diminished in similar circumstances has not been obtained.

3. The substance that is thrown out of solution is derived mainly from the antiserum.

4. The character of an antiserum depends upon two factors which are mutually independent: (1) the precipitable content; (2) its precipitability.

5. The precipitable content is indicated by the maximum precipitum obtainable from a given amount of the antiserum.

6. Its precipitability is indicated by the minimum amount of homologous proteid that will completely neutralise the precipitin in a given amount of the antiserum.

7. The solid content of precipitin antisera is increased relatively to that of natural sera.

We desire to express our indebtedness to Professor Anderson Stuart, in whose laboratory most of our work has been done, and to Dr. G. H. F. Nuttall, whose book has been of great service in giving an account of the work of other observers whose original communications we have been unable to consult.

Observations on the Development of Ornithorhynchus.

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(Abstract.)

The paper treats of certain stages in the intrauterine development of the egg of Ornithorhynchus.

The stages dealt with include the following:—

(1) An early (eight-celled) stage of segmentation.

(2) A stage manifesting a later phase of the segmentation-process.

(3) A stage of early germinal-layer formation, in which the cellular blastoderm is almost exclusively arranged in the form of a much-attenuated epithelial membrane covering part of the yolk-mass. The arrangement on the whole resembles that illustrated in fig. 22, Taf. 8, and fig. 33, Taf. 9, in R. Semon's work on "Monotreme Development."* Certain differences are, however, noticeable, more especially as regards the absence of the deeply placed nuclei figured by Semon.

(4) A stage of more advanced germinal-layer formation, characterised by the complete differentiation of the yolk-entoderm and of a lenticular mass of cells connected with the outer layer of the vesicle, which would seem to represent an early condition of "primitive knot" formation. This stage represents that of the completed "first phase of gastrulation."

(5) A stage exhibiting the characteristics of the "second phase of gastrulation." These include the presence of a fully-developed "primitive knot" which may henceforth be designated as "archenteric," owing to the formation in connection with it, by invagination, of an "archenteric," or "gastrulation-cavity." Meanwhile there has also developed—quite independently of, and

* 'Zool. Forschungsreisen in Australien,' etc., 1894, Bd. 2, Lief. 1.