THE SPECIFICITY OF ANTISERA AGAINST CRYSTALLINE SERUM ALBUMIN

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Introduction

The subject of the specificity of immunological reactions has been reviewed recently by Landsteiner (1936) and by Marrack (1938). The present communication records a quantitative investigation of the specificity of antisera prepared by injection into rabbits of crystalline horse-serum albumin and crystalline human-serum albumin. That antisera obtained after several courses of injections of whole serum will react with sera of animals of different species is a phenomenon familiar to serologists, and we considered it to be of interest to determine whether a more strictly specific antiserum might be produced if only one antigen present in serum were injected.

Our results have confirmed the loss (demonstrated by Hooker & Boyd (1934, 1936)), of apparent specificity of antisera obtained after more than one course of injections of a highly purified antigen. These authors made a quantitative study of the cross-reactions of antigen-antibody systems in which the antigens were the crystalline ovalbumins of the hen and the duck. They have reported that if rabbits were injected with hen ovalbumin, free from conalbumin, early bleedings yielded antisera which reacted only with homologous antigen, whereas subsequent bleedings gave antisera which also formed precipitates with duck ovalbumin. Their quantitative data, based on nitrogen determinations, showed that considerably more antibody was precipitated from the serum by the homologous than by the heterologous antigen.

During the course of our work, a further paper on this subject was published by Cole (1938) who has investigated the specificity of antigen-antibody systems in which the antigens were the ovalbumins of the hen, the guinea-fowl, the Amherst pheasant and the golden pheasant, and has shown that antisera against hen conalbumin react with mother liquors from guinea-fowl and pheasant crystalline ovalbumins as well as with the homologous antigen. This indicates that there are immunological relationships between the serum albumins of these birds, as conalbumin, the non-crystallizable fraction of ovalbumin, is considered to be serologically similar to serum albumin. From the details given it is not clear whether the cross-reactions reported were shown by antisera prepared after one course of injections.

In the systems we have studied, we have been unable definitely to decide whether the antisera obtained from the first bleedings differ qualitatively or

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only quantitatively in their specificity from those derived from subsequent bleedings.

METHODS

Horse-serum albumin was recrystallized twelve times by the method of Adair & Robinson (1930). We have been unable to estimate the carbohydrate content quantitatively, but the solutions obtained gave a faintly positive Molisch reaction. We have not found twelve times recrystallized albumin to be a poor antigen, as good precipitating antisera have generally been obtained after two courses of injections. Human-serum albumin was crystallized as described by Adair & Taylor (1935), but owing to the small quantities of human serum available, this protein was not recrystallized and probably contained traces of serum globulin.

Horse-serum globulin was prepared by the addition to horse serum of an equal volume of saturated ammonium sulphate solution. The precipitate obtained was dissolved and reprecipitated five times as described by Taylor *et al.* (1932). We are fully aware that the fractionation of serum by means of ammonium sulphate is not the best procedure available, but unfortunately we have not had access to an apparatus for the separation of protein fractions by means of electrophoresis (Tiselius, 1937).

All the proteins and antisera used were preserved at $-10°$ C. and were only brought to room temperature immediately before use. Rabbits were injected with 4 mg. protein daily for 5–7 consecutive days. The first injection of a course was given intraperitoneally, and the subsequent injections intravenously. The rabbits were bled from the ear vein on the eighth or ninth day after the last injection. The successive bleedings discussed in the text are distinguished by the appropriate letter of the alphabet, e.g. 2594 F indicates the antiserum obtained after six courses of injections.

A relationship between the specificity of the antisera obtained and the method of injection has been postulated by Wolfe (1935, 1936).

The antibody contents of the antisera were determined by the method of optimal proportions (Dean & Webb, 1926; Taylor *et al.* 1932), the results being recorded as optimal ratios which express the proportions by volume of antiserum and a standard 1% solution of albumin found to yield the most rapid particulation. Ring tests were made by the addition of falling dilutions of antigen to undiluted antiserum, the reactions at the interface of the two fluids being read after the tubes had stood for 35 min. at room temperature. In every case control tubes with antigen and normal rabbit serum were set up and inspected.

Precipitates for nitrogen determinations were obtained and examined as described by Adair & Taylor (1936). Horse-serum albumin was assumed to contain 15·6% of nitrogen (Adair & Robinson, 1930). The same figure has been used for human-serum albumin.

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Experiments

Our first experiments were carried out with antisera from rabbits which had received more than one course of injections of the antigen. In each case examined, antisera prepared by injection of human-serum albumin reacted with horse-serum albumin, while those against horse-serum albumin reacted with human-serum albumin, as shown by ring tests. The results of optimal proportion titrations and determinations of the ratio of precipitate nitrogen to antigen nitrogen at the optimal point, before and after absorption with heterologous antigen, are recorded in Table I.

In the case of rabbit 2714, two antisera were used, one obtained after the second course of injections with human-serum albumin, and the other a pool of the third and fifth bleedings. Neither of these antisera reacted sufficiently powerfully with the heterologous antigen to allow of a fine test, by the method of optimal proportions. A modified rough test was carried out in small tubes with 0·25 c.c. of undiluted antiserum and equal volumes of falling dilutions of antigen, dilutions being made by means of "50 dropper pipettes", described by Donald (1915). An approximate estimate of the optimal point was thus found, and absorption with heterologous antigen was carried out in the region of slight antigen excess.

In the cases of three sera, namely, antihuman-serum albumin 2594 F and 3078 B, and antihorse-serum albumin 3031 D, the reaction with the heterologous antigen was sufficiently rapid to enable a fine test to be performed, and absorption was carried out at the optimal point so determined.

The following conclusions may be drawn from the data given in Table I:

(1) More antibody is precipitated at the optimal point by the homologous than by the heterologous antigen.

(2) Less antibody is precipitated by the homologous antigen at the optimal point if the antiserum has been previously absorbed with the heterologous antigen. These two findings agree with those reported by Hooker & Boyd (1936) from their studies of the reactions of antisera prepared against crystalline ovalbumins.

(3) The sum of the amounts of antibody precipitated if the antiserum be first treated with heterologous and then with homologous antigen approximates to the amount obtained from the reaction of homologous antigen with previously unabsorbed antiserum.

(4) In every case the precipitation reaction between homologous antigen and antiserum takes place much more slowly after absorption with the heterologous antigen. As the comparative titrations were carried out simultaneously, this effect should not be due to any variation of temperature.

Similar relationships between the amounts of antibody precipitated by absorption with homologous and heterologous antigens have been observed by Heidelberger & Kendall (1934) in the case of a system in which the antigen

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Table I

Antisera	Fine tests: Heterologous		Fine tests: Homologous, Before heterologous absorption		Fine tests: Homologous, After heterologous absorption		Estimation of the nitrogen content of the precipitate: 1 Heterologous absorption		2 Homologous after heterologous absorption		Sum of (1) and (2)	3 Homologous absorption of previously unabsorbed serum		Ratio of precipitate nitrogen to antigen nitrogen		
Anti-human-albumin sera	Ratio	Time in min.	Ratio	Time in min.	Ratio	Time in min.	Mg. N per 3 c.c. anti-serum	Average per c.c.	Mg. N per c.c. anti-serum	Average per c.c.	Mg. N per c.c.	Mg. N per c.c. anti-serum	Average per c.c.	Heterologous absorption	Homologous absorption of serum previously absorbed	Homologous absorption of serum *not* previously absorbed
2714 C, E	1 : 75 (Rough only)	50	1 : 23·2	19	1 : 23·2	24	0·225 0·238	*0·077*	0·595 0·595 0·581	*0·590*	*0·667*	0·637 0·649 0·639	*0·639*	3·702	8·774	9·503
2714 B	1 : 150 (Rough)	200	1 : 49·5	43	1 : 49·5	85	0·112 0·117	*0·038*	0·530 0·515	*0·522*	*0·560*	0·578 0·574	*0·576*	3·654	8·302	9·12
2594 F	1 : 55·2	60	1 : 12·6	18	1 : 15·2	30	0·401 0·408	*0·133*	0·846 0·847	*0·846*	*0·979*	1·076 1·077	*1·076*	4·706	8·250	8·695
3078 B	1 : 61·8	30	1 : 10·5	15	1 : 12·36	20	0·510 0·546	*0·176*	1·360 —	*1·360*	*1·536*	1·635 1·621	*1·628*	7·368	10·77	10·88
Antihorse-albumin serum																
3031 D	1 : 107·1	65	1 : 12·8	10	1 : 12·8	20	0·373 0·371	*0·124*	0·878 0·859	*0·868*	*0·992*	1·055 1·093 1·077	*1·075*	8·513	7·12	8·818

was *R*-salt-azobenzidine-azo ovalbumin, which yielded an antiserum reacting with the homologous antigen and also with crystalline ovalbumin, and by Marrack & Carpenter (1938), who have investigated the reactions of type II antipneumococcal sera and certain vegetable gums.

We are unable to offer any explanation of the very considerable differences between the ratios of precipitate nitrogen to antigen nitrogen determined for the reactions between horse-serum albumin and the antihuman albumin sera. As shown in Table I, in the case of A.S. 3078 B, this ratio is 7·37, whereas the other three antisera gave much smaller values. As stated above, the absorptions with heterologous antigen and A.S. 2714 B and 2714 C and E were carried out with a slight excess of antigen, but it seems unlikely that this would cause a diminution of about 50% in the ratio. In the case of A.S. 2594 F, when absorption with heterologous antigen was carried out at the optimal point, the ratio determined was 4·71.

An alteration in the optimal ratio of antiserum with homologous antigen was observed only in the cases of A.S. 2594 F and 3078 B, in both of which sufficient antibody was removed by the heterologous absorption for its loss to be detectable by fine tests. According to our computations, heterologous absorption of the other sera recorded did not cause a reduction of total antibody sufficient to be measured by the optimal proportions method, even by a fine test. Hooker & Boyd (1936) have questioned the value of optimal proportions titrations carried out after heterologous absorption.

We then examined antisera which had been obtained after only one course of injections; the results of these experiments are recorded in Table II. In the case of fifteen rabbits injected with horse-serum albumin only one, 3075 A, produced a reasonably rapidly precipitating antiserum, and this antiserum was found to be remarkably non-specific (see Table III). A second antiserum, 3097 A, which revealed a high titre with homologous antigen when subjected to ring tests, was concentrated by means of precipitating the serum globulin by half-saturation with ammonium sulphate solution. After redissolving the precipitate, the solution obtained was dialysed against saline at 0° C. and concentrated by ultra-filtration until the protein content was approximately that of the original serum.

The ratios of precipitate nitrogen to antigen nitrogen obtained at the optimal point in the cases of A.S. 3075 A and of the concentrate of A.S. 3097 A were considerably lower than those found by Taylor *et al.* (1934) and by Kabat & Heidelberger (1937) for crystalline horse-serum albumin and its homologous antiserum.

Of eight rabbits injected with human-serum albumin, three gave reasonably good precipitating antisera; these were investigated, together with the globulin fraction of A.S. 3081 A, after this had been prepared in the same way as A.S. 3097 A.

The ratios of precipitate nitrogen to antigen nitrogen determined at the optimal point were similar to those obtained for the antihorse albumin sera

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Table II

"A" bleedings

Antisera	Fine test ratio	Mg. N absorbed from 3 c.c. antiserum with homologous antigen	Test for excess antigen in supernatant	Ratio of precipitate N to antigen N
Antihorse-albumin sera				
3075 A	1 : 96·4	0·2311 0·2746	0	5·20
3097 A (concentrate)	1 : 42·4	0·2263 (per 1·5 c.c. serum) 0·2270	0	4·10
			Average	4·65
Antihuman albumin sera				
3078 A	1 : 55·7	0·401 0·408	0	4·81
3079 A	1 : 102·8	0·257 0·248	0	5·55
3080 A	1 : 48·2	0·386 0·367	+	3·87
3081 A (concentrate)	1 : 24·7	0·297 (per 1 c.c. antiserum) 0·289	0	4·64
			Average	4·72

"B" bleedings

Antisera	Fine test ratio	Mg. N absorbed from 1 c.c. antiserum with homologous antigen	Test for excess antigen in supernatant	Ratio of precipitate N to antigen N
Antihorse-albumin sera				
3075 B	1 : 21·4	1·49 (per 2 c.c. antiserum) 1·55	0	10·42
3076 B	1 : 58·7	0·385 (per 2 c.c. antiserum) 0·389	0	7·27
3031 D	1 : 12·8	1·055 1·093 1·077	+	8·82
			Average	8·83
Antihuman albumin sera				
3078 B	1 : 10·5	1·635 1·621	+	10·88
3080 B	1 : 13·07	1·266 1·297	0	10·7
3083 B	1 : 91·02	0·4903 (per 3 c.c. antiserum) 0·5295	0	9·94
2714 C, E	1 : 23·2	0·637 0·649 0·633	+	9·50
2714 B	1 : 49·5	0·578 0·574	+	9·12
2594 F	1 : 12·6	1·076 1·077	+	8·69
			Average	9·80

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3075 A and 3097 A (Table II). These values of the ratios for antisera obtained after one course of injections are probably less accurate than those published by Taylor *et al.* (1934) and by Kabat & Heidelberger (1937), as, in some cases, the amount of nitrogen present was small. We are, however, convinced that experimental errors could not account for the differences between our determinations and those of previous authors.

The antihuman-albumin sera 3078 A, 3079 A, 3080 A and 3081 A did not react with horse-serum albumin or with any of the heterologous antigens tested except in the case of the concentrate of 3081 A, which appeared to give a positive ring test with a 3·5% solution of horse-serum albumin (Table III). We do not feel justified in emphasizing this exception, as we had no preparation of normal rabbit globulin and were unable to set up controls similar to those usually carried out with normal rabbit serum.

Table III

Antihorse-albumin-sera	Horse albumin 1%	Horse serum	Human albumin 5·7%	Human serum	Horse globulin 4·0%	Ox serum	Pig serum	Sheep serum
3075 A	1 : 5,120	1 : 8,000	1 : 2	1 : 8	1 : 128	1 : 500	1 : 64	1 : 128
3076 A	1 : 640	1 : 2,000	1 : 1	1 : 64	1 : 32	1 : 32	0	0
3095 A	1 : 1,280	1 : 5,120	1 : 1	0	1 : 32	0	0	0
3096 A	1 : 640	1 : 5,120	0	0	1 : 8	0	0	0
3097 A	1 : 10,240	1 : 5,120	0	0	1 : 128	0	0	0

Antihuman-albumin sera	Human albumin 1%	Human serum	Horse albumin 14·0%	Horse serum	Horse globulin 4%	Ox serum	Pig serum	Sheep serum
3078 A	1 : 2,048	1 : 16,000	0	0	0	0	0	0
3079 A	1 : 2,048	1 : 8,000	0	0	0	0	0	0
3080 A	1 : 2,048	1 : 8,000	0	0	0	0	0	0
3081 A	1 : 2,048	–	0	–	–	–	–	–
3081 A concentrate	+	–	+ (3·5%)	–	–	–	–	–
3082 A concentrate	+	–	0	–	–	–	–	–
3083 A concentrate	+	–	0	–	–	–	–	–

+ = Positive, but titre not determined. – = Not tested. 0 = Tested, but reaction negative.

The rabbits whose sera has been examined after one course of injections were reinjected with the appropriate antigens and the antisera obtained were investigated. The results are recorded in Table II. It may be observed that the antihorse-serum albumin sera, 3075 B and 3076 B, gave ratios of precipitate nitrogen to antigen nitrogen at the optimal point similar to those reported by Taylor *et al.* (1934) and Kabat & Heidelberger (1937). This value is considerably larger than that shown by the first bleedings. An analogous increase in this ratio was found for the antihuman-albumin sera 3078 B, 3080 B and also for 2714 B, 2714 C and E, and 2594 F.

It may be mentioned that, although some of the values for equivalence point ratios given by Kabat & Heidelberger (1937) refer to antisera obtained from a first bleeding, the rabbits used in their experiments are reported to have received sixteen to twenty-four injections during each course; the antisera are

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therefore not necessarily comparable with those obtained after six or eight injections. The total amount of protein, however, injected by Kabat & Heidelberger (1937) in twenty-four doses did not differ greatly from that given by ourselves in eight.

Baumgartner (1937) has reported that in the system crystalline hen ovalbumin and its homologous antiserum, the ratio of precipitate nitrogen to antigen nitrogen in the equivalence zone is sometimes greater in the case of young animals (8–17 weeks old) than for those of adults. The values for the ratios given by this author seem to be unrelated to the number of courses of injections given.

The results of investigations of the specificity of antisera obtained from the first and second bleedings are recorded in Tables III and IV. All the antisera from rabbits which had received one course of injections of twelve times recrystallized horse-serum albumin reacted with six times precipitated horse-serum globulin. This might indicate either that globulin and albumin possessed common antigenic groupings or that the globulin was contaminated with albumin. Specificity tests carried out by Dr van den Ende on uterine strips sensitized with six times precipitated globulin and twelve times crystallized horse-serum albumin suggest contamination.

A.S. 3075 A reacted with all the antigens with which it was tested; a positive reaction with human-serum albumin and human whole serum was given only with relatively high concentration of antigen. The antihuman-albumin sera obtained after one course of injections were apparently more specific. As recorded in Table IV, antisera obtained after a second course had undergone a considerable diminution in specificity.

Table V gives the results of ring tests carried out after the antisera which reacted with both homologous and heterologous antigens had been absorbed with human serum and with horse-serum albumin. The findings are similar to those of previous authors (Landsteiner & van der Scheer, 1936; Marrack & Carpenter, 1938; Cole, 1938; Kabat & Heidelberger, 1937) in that absorption with homologous antigen at the optimal point removed all reactivity with heterologous antigen. It has been asserted by Kabat & Heidelberger (1937) that the persistence of the reaction with homologous antigen after homologous absorption at the optimal point is due to contamination with globulin of the albumin used for immunization, so that antibody to this protein is also produced and gives a residual reaction due to globulin-antiglobulin.

The differences in specificity between antisera obtained after one course of injections and those from subsequent bleedings may be quantitative, in that the more feeble heterologous reactions are brought about by antibodies which, after only one course, are present in too low a concentration to yield visible precipitates in the ring test within a reasonable period of time.

We attempted to concentrate the antibody by preparing globulin fractions from a pool of six antihuman-albumin sera obtained after one course of injections, using the method of Adair & Taylor (1936). A 6·0% solution of the

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Table IV

Antihorse-albumin sera	Horse albumin 1%	Horse serum	Human albumin 1%	Human serum	Horse globulin 4·22%	Ox serum	Pig serum	Sheep serum
3075 B	1 : 10,240	1 : 32,000	1 : 2,560	1 : 8,000	1 : 256	1 : 32,768	1 : 512	1 : 8,192
3076 B	1 : 5,120	1 : 32,000	1 : 1,280	1 : 8,000	1 : 256	1 : 16,384	1 : 4,096	1 : 8,192

Antihuman-albumin sera	Human albumin 1%	Human serum	Horse albumin 1%	Horse serum	Horse globulin 4·22%	Ox serum	Pig serum	Sheep serum
3078 B	1 : 10,240	1 : 16,000	1 : 5,120	1 : 4,000	1 : 16	1 : 32,768	1 : 16,384	1 : 8,192
3079 B	1 : 10,240	1 : 16,000	1 : 2,560	1 : 4,000	1 : 16	1 : 8,192	1 : 8,192	1 : 4,096
3080 B	1 : 10,240	1 : 16,000	1 : 5,120	1 : 4,000	1 : 32	1 : 32,768	1 : 8,192	1 : 8,192
3081 B	1 : 5,120	1 : 16,000	1 : 1,280	1 : 2,000	1 : 2	1 : 64	1 : 256	1 : 128
3082 B	1 : 5,120	1 : 32,000	1 : 1,280	1 : 4,000	1 : 8	1 : 8,000	1 : 2,040	1 : 256
3083 B	1 : 10,240	1 : 32,000	1 : 1,280	1 : 4,000	1 : 8	1 : 256	1 : 256	1 : 256

Table V

Antisera	Unabsorbed		After homologous absorption			After heterologous absorption			After heterologous followed by homologous absorption		
Anti-human-albumin sera	Human albumin 1%	Horse albumin 1%	Human albumin 1%	Horse albumin 1%	Neat antiserum	Human albumin 1%	Horse albumin 1%	Neat antiserum	Human albumin 1%	Horse albumin 1%	Neat antiserum
2714 C, E	1 : 5,120	1 : 1,280	+	+	+	1 : 5,120	+	+	+	+	+
2714 B	1 : 5,120	1 : 2,560	1 : 160	0	+	1 : 5,120	+	+	1 : 80	+	+
2594 F	1 : 10,240	1 : 5,120	1 : 160	1 : 8 (of 6%)	+	1 : 10,240	1 : 2 (of 6%)	+	1 : 80	1 : 8 (of 6%)	+
3078 B	1 : 10,240	1 : 2,560	1 : 20	0	0	1 : 10,240	1 : 2 (of 6%)	0	1 : 320	0	+
Antihorse albumin serum	Horse albumin 1%	Human albumin 1%	Horse albumin 1%	Human albumin 5·72%	Neat antiserum	Horse albumin 1%	Human albumin 5·72%	Neat antiserum	Horse albumin 1%	Human albumin 5·72%	Neat antiserum
3031 D*	1 : 5,120	1 : 2,560	1 : 10	1 : 2	0	1 : 2,560	1 : 2	+	1 : 10	0	+

* Antigen used for immunization was only 4 times crystallized.

main euglobulin fraction gave an optimal ratio of 1 to 32, when titrated against homologous antigen. Ring tests with the undiluted euglobulin gave rather dubious reactions with 3·5% horse-serum albumin and with a 1 in 10 dilution of pig serum, but not with the other heterologous antigens tested. A 9·43% solution of "euglobulin 2" (the fraction whose solubility in ammonium sulphate is slightly lower than that of pseudoglobulin) gave a ratio of 1 in 24 with homologous antigen, and ring tests again showed traces of precipitate with 3·5% horse-serum albumin, with a 1 in 10 dilution of pig serum and also with a 1 in 10 dilution of horse serum. A 10% solution of pseudoglobulin was found to react very slowly with homologous antigen in the optimal proportions titration, giving a precipitate at a ratio of 1 in 96 in about 4 hr. Ring tests did not show any reactions between the pseudoglobulin fraction and the heterologous antigens. The crude method of fractionation employed did not give satisfactory results, as, although the optimal proportions titrations of the euglobulin solutions showed that an appreciable concentration of antibody had been brought about, the results of the ring tests were too meagre to serve as a basis for a decision as to the strict specificity of the antisera obtained after one course of injections. It is generally agreed that ring tests tend to be unreliable if a concentrated antigen solution be used.

DISCUSSION

The findings recorded regarding the loss of specificity of antisera obtained after more than one course of injections of crystalline serum albumin support the conclusion of Hooker & Boyd (1936) and of Cole (1938) that the injection of a highly purified antigen may give rise to a number of distinct precipitins. The twelve times crystallized horse-serum albumin used in our experiments is probably a mixture of closely related components rather than a single chemical individual (Sørensen, 1930), but this consideration alone will not explain the apparent loss of species specificity of the antisera obtained after more than one course of injections of the protein; it would seem to be necessary to postulate that similar antigenic components are present in the serum albumins of different species.

The differences determined between the ratios of precipitate nitrogen to antigen nitrogen for antisera derived from a first bleeding and those from subsequent bleedings are of interest in the light of the hypothesis put forward by Boyd & Hooker (1934) who relate the equivalence point ratio by weight of antibody to antigen with the molecular weight of the antigen. These differences in the ratio may indicate that there is a true qualitative change in the antibody during prolonged immunization.

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SUMMARY

Antisera prepared in the rabbit by injection of crystalline horse-serum albumin or crystalline human-serum albumin show a considerable diminution in specificity after more than one course of six to eight injections has been given.

The ratio of precipitate nitrogen to antigen nitrogen at the optimal point is smaller in antisera obtained after one course of injections than in antisera derived from subsequent bleedings.

In the case of antisera obtained from first bleedings, the ratio of precipitate nitrogen to antigen nitrogen determined at the optimal point with homologous antigen is of approximately the same value in both the antigen-antibody systems studied. The increase in the values of the ratios for antisera from subsequent bleedings is also the same for both systems.

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