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Physical Factors affecting Maximum Precipitation of the BSA-AntiBSA Fowl System*

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Summary. Studies were made ascertaining the effects of temperature and length of incubation period on the amount of precipitate formed in the BSA-antiBSA fowl serum system. The specific factors considered were centrifugal temperature, temperature of incubation and length of incubation. Reactions were analysed for the entire precipitin curve, using doubling dilutions of antigen, and also for the region of equivalence using intervals of 1 μg. nitrogen.

Reactions mixtures were incubated at 37° for 3 hours and then centrifuged at 22° or at 4° and the precipitate was analysed for total N precipitated. In addition, secondary incubation periods, following this initial incubation treatment of 3 hours at 37°, were made. The secondary periods were either 18, 66 or 118 hours at 4° or 18 hours at 37° and were followed by centrifugation at either 22° or 4°.

Evaluation of the data showed centrifugation at 22° gave higher amounts of precipitate than at 4° in all cases and the differences were statistically significant in most of the instances. In eighteen of the twenty sera tested, precipitation was at a maximum after 3 hours' incubation at 37° and warm centrifugation in contrast to additional incubation periods and/or cold centrifugation.

INTRODUCTION

The establishment of the quantitative technique for the determination of antibody nitrogen by Heidelberger *et al.* (1929, 1933) was an important advance in serology. In perfecting this procedure it has been important to standardize certain chemical and physical conditions in order to be able to secure reliable and comparable results. Such factors as salt concentration, *p*H, temperature, and length of incubation period have been studied; data relating to the effect of temperature and length of incubation period are quite meagre. These factors have been studied for horse and rabbit antisera, but it has been shown that the reactions of fowl antisera may differ from those of horse and rabbit antisera in a number of respects. We have therefore thought it advisable to study directly the effects of temperature and incubation period upon precipitation of fowl antiserum with homologous antigen.

Heidelberger *et al.* (1933), using the azoprotein-anti-azoprotein rabbit system, reported equal precipitation whether the incubation period was 2 hours at 37° followed by overnight in the ice-box or in the ice-box throughout. The same authors (1935c) stated that some rabbit antisera occasionally gave more specifically precipitable N per ml. when allowed to stand at 0° for 48 instead of 24 hours. Therefore their determinations were allowed to stand at 0° for 2 days except in the inhibition zone, for which 4 days were allowed.

Heidelberger and Kendall (1935a, 1935b), using a pneumococcus polysaccharide

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anti-pneumococcus polysaccharide horse system, found that greater amount of precipitate formed when the temperature was 0° throughout incubation rather than 37°. Heidelberger *et al.* (1942) found that temperature greatly affected cross-reactions. Using an anti type VIII pneumococcus horse antiserum against type III polysaccharide they found considerably more precipitate resulted when the temperature of the reaction was 0° throughout, rather than 37° throughout, or 37° for 2 hours followed by chilling overnight in the refrigerator.

The usual procedure followed by many workers using the rabbit system is composed of an initial incubation period of 1 to 3 hours at room temperature or 37° followed by incubation for 1 to several days at 4°. This is followed in turn by centrifugation in a refrigerated centrifuge.

Wolfe and his colleagues have been using the fowl for precipitin production for a

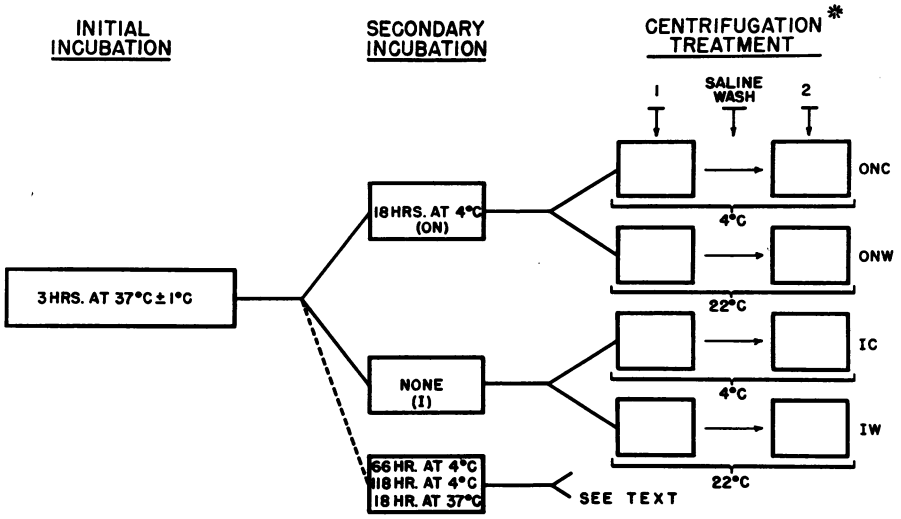

FIG. 1. Experimental Design.

number of years. Not only have they found that fowl antisera react differently from rabbit antisera to salt concentration (Goodman *et al.*, 1951), but have also noted certain differences in aging (Gengozian and Wolfe, 1957) of antisera not known to occur among mammalian species. Munoz and Becker (1952) indicate no significant difference between the amount of precipitate obtained with fowl anti-rabbit globulin serum incubated for 3 days at room or refrigerator temperature.

The purpose of this paper is to report the effects of temperature and length of incubation period on precipitation of a BSA-antiBSA fowl system.

EXPERIMENTAL METHODS

Pooled antisera produced in male fowls were used in these experiments. The birds were given a single inoculation of 40 mg. bovine serum albumin* (BSA) per kilogram body weight and bled 8 days later after a starvation period of 18 to 24 hours. A preliminary titration of the individual sera allowed pooling of sera of similar strength; different pools

* Purchased from Pentex Company.

were classified as weak, medium or strong precipitating sera. The sera were prepared in the usual manner and stored at −20°.

The antibody-N was determined by procedures devised by Heidelberger *et al.* (1933). Since Goodman *et al.* (1951) have shown that chicken antisera react optimally in about 8 per cent. saline, this concentration was employed. Reaction mixtures contained 0·25 ml. of antiserum in a total volume of 1·0 ml. Nine sera were used with doubling antigen dilutions (1 to 256 μg N/ml. of reaction mixture) including the regions of antibody excess, equivalence and antigen excess; eight antisera were reacted in the region of equivalence only. Five to seven different antigen concentrations at intervals of 1 μg N were used.

Most tests were run in duplicate, but several were run in triplicate. In general, four identical sets* of reaction mixtures were set up simultaneously for each antiserum tested, one of which was carried through each of the four treatments described in Fig. 1. All four sets were first given a primary incubation period of 3 hours at 37° ±2°. Two of the four sets were then centrifuged immediately for 30 minutes, one at 22° (IW), and the other at 4° (IC). The other two sets were given an additional 18 hours' incubation at 4°

TABLE 1

VARIANCE DISTRIBUTION FOR SERUM PC-5. THE OUTLINED METHOD WAS APPLIED TO ALL SIMILAR SETS; RESULTS ARE SUMMARIZED IN TABLE 2

Source	*Degrees of freedom*	*Sum of squares*	*Mean square*	*F ratio*
1. Total	107–6*	104,097		
2. Blocks** ..	3	5,277		
3. WxC	1	408	408	13·94
4. ONxI	1	4,800	4,800	164·00
5. Interaction ..	1	69	69	2·36
6. Within blocks ..	98	98,820		
7. Dilution	8	96,186		
8. Replicate error ..	90***	2,633	29	

* Number of tubes destroyed by breakage, drying up, or boiling over.
** A group of tubes treated similarly.
*** Three replicates.

(ON), and were then centrifuged for 30 minutes, one at 4° (ONC), the other at 22° (ONW). All precipitates were washed with 5 ml. of an 8 per cent saline solution; the temperature of the saline used for each set was the same as the centrifugation temperature for that set. All sets were then centrifuged for an additional 30 minutes at the same temperature as the original centrifugation for each set. After these procedures, nitrogen content of the precipitates was determined by the colorimetric method of Johnson (1940).

For each serum the data consisted of the total nitrogen values for a series of tubes treated according to the above schedules. Within each of the four treatments was an antigen dilution series (usually 9 concentrations; see Fig. 2), and at each point of the dilution series 2 or 3 replicates were entered. Variation among the nitrogen values derived from a single serum was analysed into variance-components and these were compared by means of the F ratio. An example of the separation of variance-components and comparisons is shown in Table 1.

* Whole curve set=9 doubling dilutions of antigen plus control (no antigen) in duplicate or triplicate. Equivalence zone set=5 to 7 antigen concentrations at one gamma intervals plus control (no antigen).

In such an analysis, items 3 and 4 measure the effects of the main treatments ind dently, centrifugation at 22° versus centrifugation at 4° and immediate centrifu versus centrifugation after overnight incubation. Item 5 measures the tendency f two main factors, temperature of centrifugation and length of incubation, to in and produce effects not predictable from the effects of the main factors acting ind dently and additively. Item 7 is a variance component introduced by the differe antigen concentration in the series of doubling dilutions used. It is of no interest he

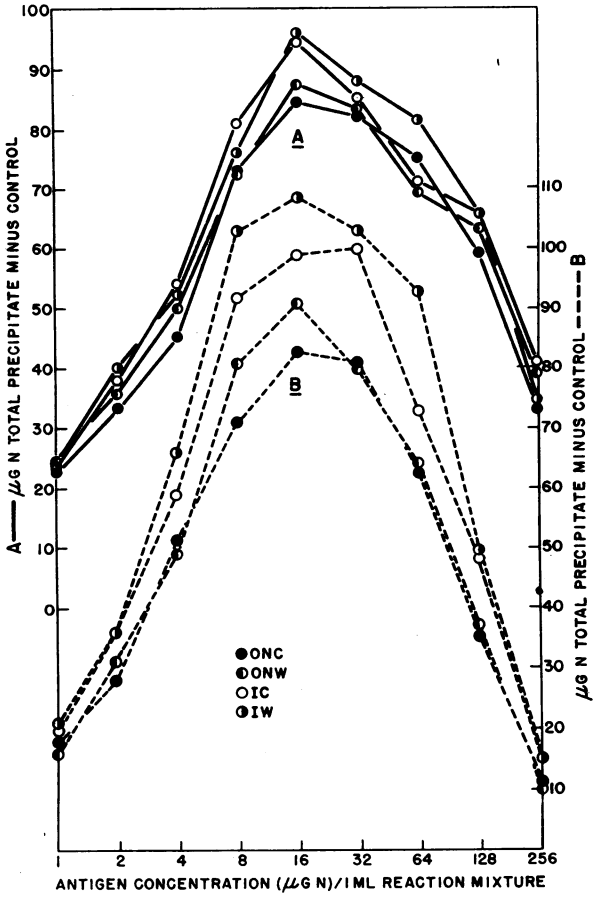

FIG. 2. Effect of temperature and time on precipitate formation in a BSA-antiBSA fowl system. Curve A————PC-6; Curve B- - - - -PC-5. ONC = secondary incubation+centrifugation at 4°; ONW = secondary incubation+centrifugation at 22°; IC = no secondary incubation+centrifugation at 4°; IW=no secondary incubation+centrifugation at 22°.

is subtracted from the within-blocks variance to leave the error term. It might be p out that a small number of tubes (approximately four or five) were lost in nitroge because of accidents, usually breakage while centrifuging, excessive evaporation the protein digestion process, etc. The value entered for each lost tube was the m the other replicate(s) at the same dilution in the same block. The number of the so replaced was subtracted from the number of degrees of freedom for error.

For each serum tested, an analysis as outlined in Table 1 was completed. The results of these are summarized in Table 2, which gives the conditions leading to maximum N precipitated for each of the single sera, along with the level of statistical significance for each of the main-factor comparisons and for the interaction. Full analyses of variance for each serum are not included here because of the great bulk involved; all would be similar to the one shown in Table 1.

Several additional experiments were performed for periods longer than 24 hours or for an incubation period longer than 3 hours at 37°. The details of these latter experiments will be described with the results.

RESULTS

Figs. 2A and 2B present graphically the data secured from 2 antisera. The majority of sera gave reactions similar to PC-6 (Fig. 2A). The greatest amount of precipitate resulted from primary incubation for 3 hours at 37° plus centrifugation at room temperature, and the least amount from secondary incubation for 18 hours followed by centrifugation at 4°. The differences in the amount of precipitate were least in the regions of extreme antibody and antigen excess. Fig. 2B shows graphically the data for antiserum PC-5. The differences between the 3- and the 3 plus 18-hour incubation periods were statistically significant, the 3-hour period resulting in the greater precipitation.

Table 2 records the factors that resulted in maximum precipitation and the results of

TABLE 2

ANALYSES OF FACTORS RESULTING IN MAXIMUM PRECIPITATION OF BSA-ANTIBSA FOWL SYSTEM

	Factors resulting in greatest precipitation: statistical significance			*Inter-action significance*
Serum No.	*Incubation period 3 hrs. at 37° C(I) vs. additional 18 hrs. (ON) at 4° C.*	*Centrifugation treatment— 30 min. at 4° C. (C) or 30 min. at 25° C (W).*	*Combination of variables giving greatest precipitation*	*Secondary incubation and centrifugation treatment*
Doubling dilutions (1 μg. to 256 μg.) including regions of ab excess, equivalence and antigen excess.				
PC1	I[1] ++[2]	W[1] NS[2]	WI[1]	NS[2]
PC2	I ++	W NS	WI	NS
PC2F[3]	I ++	W NS	WI	NS
PC3	I NS	W ++	WI	++
PC4	I ++	W ++	WI	NS
PC5	I ++	W ++	WI	NS
PC6	I ++	W NS	WI	NS
PC7	I ++	W ++	WI	++
PC7F[3]	I ++	W NS	CI	NS
Equivalence region only—1 gamma N antigen intervals.				
PC2	ON NS	W ++	WI	NS
PC5	ON NS	W +	WI	NS
PC8	I ++	W NS	WI	NS
PC9	I ++	W NS	CI	NS
PC10	I ++	W ++	WI	NS
PC11	I +	W +	WI	++
PC12	I +	W ++	WI	++
PC13	I ++	W ++	WI	+

[1] Factor giving greater precipitate.
[2] ++ = significant 1 per cent level; + = significant 5 per cent level; NS = not significant.
[3] Gamma-globulin fraction, all others whole sera.

the statistical analyses of the data for the nine antisera tested at doubling dilutions that included regions of antibody excess, equivalence and antigen excess as well as of the data for the eight antisera tested in the equivalence region alone.

One of the main factors considered was length of incubation period. The total amount of precipitate after 3 hours' incubation (I) at 37° ±2° (plus warm or cold centrifugation) was greater than after an additional (secondary) 18-hour period (ON) at 4° ±2° (plus warm or cold centrifugation) with fifteen of the seventeen antisera. With thirteen of these the differences were statistically significant at the 1 per cent level and with two at the 5 per cent level. Two antisera tested only in the equivalence zone gave apparently but not significantly greater precipitation after overnight incubation than after 3 hours' incubation.

TABLE 3

SUMMARY OF RESULTS OF VARYING CONDITIONS DURING THE SECONDARY INCUBATION PHASE

Antiserum	*Treatments compared*	*Factors resulting in greatest precipitation: statistical significance*		*Combination giving maximum precipitate*
		Incubation period	*Centrifugation temperature W=30 min. at 22° C=30 min. at 4°*	
PC-307	I[1] vs. 18 hrs. at 4°	I ++[2]	W +	W I
	I vs. 66 hrs. at 4°	I ++	W +	W I
	I vs. 118 hrs. at 4°	I ++	W NS	W I
	18 hrs. at 4° vs. 66 hrs. at 4°	18 NS	W ++	W 18
	18 hrs. at 4° vs. 118 hrs. at 4°	18 ++	W NS	W 18
	66 hrs. at 4° vs. 118 hrs. at 4°	66 ++	W NS	W 66
PC-320[3]	I vs. 18 hrs. at 37°	I ++		
	I vs. 18 hrs. at 4°	I ++		
	18 hrs. at 4° vs. 18 hrs. at 37°	18 hrs. at 4°+		

[1] I refers to an initial primary incubation of 3 hours at 37.°

[2] ++ = significant at 1 per cent level; + = significant at 5 per cent level; NS = not significant.

[3] Centrifugation was only at 22°.

The effect of centrifugation temperature was also considered. For all seventeen sera the warmer temperature resulted in greater precipitation (data for 3 and 18 hours' incubation combined at each temperature); for eight of these results were significant at the 1 per cent level, for two at the 5 per cent level and for the remaining seven the differences were not significant.

An experiment was then performed to study the effects of periods of incubation longer than those described in the above experiments. This was done in order to ascertain whether the loss of precipitate is a continuous time function of incubation. In addition to primary incubation for 3 hours or for 3 hours plus an 18-hours' secondary incubation (employed in the preceding experiments), secondary incubations for 42, 66 and 118 hours were investigated. Table 3 records the data for one (PC307) of three antisera so tested. This antiserum was set up in eight sets at one time, all sets being primarily

incubated at 37°±2° for 3 hours. Two of these sets were centrifuged immediately and two each of the six others received secondary incubation periods of 18, 66 or 118 hours at 4°. Of the two sets receiving each secondary incubation period, one was centrifuged at 22° (W) and the other at 4° (C). This resulted in eight different treatment combinations of incubation time and centrifugation temperature. As shown in Table 3, the shorter the secondary incubation period, the greater the amount of precipitate. Of the eight treatment combinations, that with a primary incubation period alone and warm centrifugation gave the greatest amount of precipitate, that with the longest period of incubation (118 hrs.) and cold centrifugation gave the least.

Lastly was tested the effect of maintaining the reaction at the constant temperature of 37° ±2° for an 18-hour secondary incubation period. This treatment was compared with (1) a primary incubation of 3 hours only and (2) an 18-hour secondary incubation period at 4°. These reaction mixtures were then centrifuged at 22°. The amount of precipitated nitrogen was greatest after the primary incubation period. When the secondary incubation was maintained at 37° ±2° there was a greater resolution of the precipitate than when the temperature was maintained at 4°. The amount of precipitated nitrogen after the cold secondary incubation period was significantly greater (5 per cent level) than that found after the warm secondary incubation period.

DISCUSSION

The effects of temperature and length of incubation period upon precipitation in the fowl antiserum-bovine serum albumin system have been studied. The amount of precipitate after a 3-hour incubation period at 37° ±2°, followed by centrifugation at 22° or 4°, was compared with amounts formed after longer incubation periods followed by warm or cold centrifugation. Our results are different from those found by other workers who used antisera produced in other species (rabbit, horse) and different antigens (egg albumin and pneumococcus polysaccharide). Two kinds of experiments were performed. One involved nine doubling serial dilutions of antigen, including the regions of antibody excess, equivalence and antigen excess, the other dilutions covering only the region of equivalence (five to seven antigen dilutions at one-microgram intervals). Twenty different antisera were tested. In eighteen of the twenty experiments, precipitation was greatest after the 3-hour incubation period followed by warm centrifugation. Longer incubations at either high or low temperatures resulted in decreased final precipitation which, of course, indicated a resolution of the original precipitate.

Statistical analyses of the effects of temperature and length of incubation were made. Changes in either or both of these variables were followed by differences in quantity of final precipitate that were statistically significant in a majority of the experiments. Warm centrifugation (22°) following a primary or secondary incubation period usually resulted in greater precipitation than did centrifugation at low (4°) temperature. Also, the shorter incubation period usually gave greater precipitation.

We cannot find reasons for the re-solution of some of the precipitate. If the precipitate that forms during the initial incubation is entirely specific, then the precipitate dissolving is part of the specific antigen-antibody complex. Two explanations may account for the phenomenon. It is possible that the initial aggregation of the specific antigen-antibody complexes entraps non-specific substances. These substances are then released as incubation continues. When whole serum was used the non-specific material could be a

number of different substances, but when the gamma globulin fraction alone was used the substances coprecipitating would have to be non-antibody globulin.

Another possible explanation is that the antiserum consists of univalent and divalent antibodies. The complex formed by the univalent antibody is entrapped by the precipitating divalent antibody-antigen complex. After a period of equilibration the univalent antibody-antigen complexes 'break away' from the lattice-like complex formed by the divalent antibodies. That fowl antiserum contains a mixture of divalent and univalent antibodies has been recently suggested by Banovitz, Singer and Wolfe (1959).

The data presented show the need for standardization of the Heidelberger technique when using fowl antisera. Apparently it is not necessary to use a refrigerated centrifuge for the quantitative precipitin method. Maximum precipitation occurs after 3 hours of incubation at high temperatures (37°) followed by warm (22°) centrifugation. Since a high salt concentration is necessary with the fowl system, contamination at these high temperatures is not a problem.

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