from those of either constituent heated separately, indicates that combination between particles of both fractions occurs during heating. The appearance of active disulphide groups indicates at least an early stage of denaturation, so that the large colloidal complexes formed during heating are formed of at least partly denatured protein particles. The combination of protein particles to form these complexes thus coincides with a change of their original structure.

Recently Van der Scheer, Wyckoff and Clarke (1941) arrived at similar conclusions from their electrophoretic studies of heated horse serum, which showed that a new colloidal aggregation product resulting from denaturation of components of the serum is formed at the expense of both the albumin and the globulin.

SUMMARY.

The effects of heating normal rabbit serum albumin and euglobulin fractions separately and together are described. When a mixture of the two fractions is heated a product is formed with properties different from those of either fraction heated separately. This product is a complex formed by the two fractions uniting as they undergo denaturation.

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EFFECT OF HEAT ON FLOCCULATING ANTIBODIES OF RABBIT ANTISERA.

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EARLIER workers have found that antisera to different antigens vary in their resistance to heat. In general it has been found that bacterial somatic agglutinins lose their flocculating power with less heating than flagellar agglutinins. For example, Jones (1927) showed that flagellar agglutinin to hog-cholera bacillus still agglutinated after the antiserum was heated at 90° C. for 20 minutes, whereas somatic agglutinin did not after the same time at 75° C. It has been also shown that some antisera which have lost their ability to agglutinate after heating for several minutes at 70–80° C. still combine with bacteria to give the phenomenon of inhibition (Eisenberg and Volk, 1902 ; Jones, 1928). This ability to inhibit is destroyed at 90° C., i.e. approximately the temperature needed for destruction of flagellar agglutinins.

Bawden and Pirie (1938*b*) showed that plant viruses with rod-shaped particles form with their antisera fluffy, open floccules similar to those formed by the agglutination of bacterial flagellar antigens, whereas those with spherical (or almost spherical) particles form dense granular precipitates similar to those formed by the agglutination of bacterial somatic antigens. When antisera to these two types of viruses are heated, they behave in the same way as antisera to the two types of bacterial antigens. Strong antisera to rod-shaped tobacco mosaic virus flocculate until heated for 10 minutes at 90° C., whereas antisera to the spherical tomato bushy stunt virus do not flocculate after heating for 10 minutes at 75° C. This paper describes experiments made to investigate the causes underlying the differences in the apparent behaviour of different antisera on heating.

METHODS AND MATERIAL.

The antigens used were human serum globulin and albumin, a strain of pea nodule bacteria (*Rhizobium leguminosarum*) and purified preparations of the following plant viruses: tobacco mosaic, potato "X," tomato bushy stunt and tobacco necrosis.

The preparations of plant viruses were made by precipitation methods and kindly supplied by Mr. F. C. Bawden.

Human globulin was prepared by half saturating human serum with ammonium sulphate. The precipitate was dissolved in water and dialysed. NaCl was then added up to 0·9 per cent. to dissolve the precipitate formed during dialysis. Albumin was precipitated by full saturation with ammonium sulphate of the filtrate remaining after the precipitation of globulin. This precipitate was dissolved in water and dialysed, and NaCl to 0·9 per cent. added.

Suspensions of pea nodule bacteria were prepared by washing the cultures grown on agar slopes with 0·9 per cent. NaCl. The bacteria were then killed with 1 per cent. formaldehyde and washed with NaCl solution.

The antisera were prepared by injecting rabbits intravenously twice a week with solutions or suspensions of the antigens. From 4 to 6 injections in all were given and the rabbits were bled 8–10 days after the last.

Most of the antibodies in the antisera to all these antigens precipitated out with the euglobulin fraction. These fractions were precipitated with 1/3 saturated ammonium sulphate. The precipitate was filtered off, dissolved in 0·9 per cent. NaCl and dialysed against 0·9 per cent. NaCl solution until the test for SO_4'' with $BaCl_2$ was negative.

The fractions of normal rabbit serum used in this work and the methods of heating were the same as those described in the previous paper (Kleczkowski, 1941).

Flocculation tests were made by mixing 1 ml. of antigen solutions with either 1 ml. of antiserum solutions or 1 ml. of antiserum euglobulin solutions. The tubes were immediately placed in a water bath at 50° C. The appearance of floccules was taken as a positive result. The ability of heated solutions to combine with antigen without causing flocculation was tested by their ability to inhibit flocculation. The solutions to be tested were mixed with

antigen solutions and incubated for 3 hours at 50° C., when 0·1 ml. of a suitable dilution of unheated antiserum was added. The absence of flocculation was taken as evidence of inhibition.

RESULTS.

The effect of heating antisera to tobacco mosaic virus and tomato bushy stunt virus.

The results shown in Table I illustrate differences in the behaviour of tobacco mosaic virus and bushy stunt virus antisera after heating. The precipitating power of bushy stunt antiserum is completely destroyed by heating for 10 minutes at 75° C., but the ability to combine with antigen is preserved; this is shown by the inhibition of flocculation when unheated antiserum is subsequently added. The inhibition is specific; it is not caused by incubation with either heated normal serum or heated heterologous antisera. The inhibition is more pronounced after heating for 10 minutes at 80° C. (higher "inhibition titre") and it still occurs after heating for 10 minutes at 85° C. It is destroyed by heating for 10 minutes at 90° C.

TABLE I.—*The Effect of Heating Antisera to Tobacco Mosaic and Bushy Stunt Viruses for 10 Minutes at Different Temperatures.*

The antisera were heated at a dilution 1/10 in physiological saline. The antigen solutions were used at 0·0025 per cent. for tobacco mosaic virus and 0·005 per cent. for bushy stunt virus. 1 ml. of antigen solution was added to a series of tubes each containing 1 ml. of antiserum at varying dilutions. + indicates precipitation and − no precipitation.

After 3 hours 0·1 ml. of control antiserum at a dilution 1/8 was added to the tubes where there was no precipitation. i indicates that there was still no precipitation (inhibition), and o that a precipitate was formed (no inhibition).

Temperature of heating.	Dilution of the antisera.													
	Tobacco mosaic virus.							Bushy stunt virus.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
Unheated	+	+	+	+	+	+	−	+	+	+	+	+	+	−
							o							o
75° C.	+	+	+	+	+	−	−	−	−	−	−	−	−	−
						o	o	i	i	i	i	o	o	o
80° C.	+	+	+	−	−	−	−	−	−	−	−	−	−	−
				o	o	o	o	i	i	i	i	i	o	o
85° C.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	o	o	o	o	o	o	o	i	i	o	o	o	o	o
90° C.	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	o	o	o	o	o	o	o	o	o	o	o	o	o	o

By contrast, the precipitating power of tobacco mosaic virus antiserum is only slightly diminished by heating for 10 minutes at 75° C., and further diminished, but not destroyed, by heating at 80° C. Sera with higher titres are only inactivated after 10 minutes at 90° C. Thus there are two obvious differences in the behaviour of the two antisera. The bushy stunt antiserum

loses its ability to precipitate at a lower temperature than tobacco mosaic antiserum, but it then inhibits precipitation, whereas no such phenomenon can be observed with the heated tobacco mosaic antiserum. The amount of heat necessary to inactivate tobacco mosaic antiserum is apparently the same as that required to destroy the ability of bushy stunt virus antiserum to combine with antigen.

The influence of dilution of antisera in saline and in protein solutions on the effect of heating.

The results of experiments given in Tables II and III show that diluting antisera to tobacco mosaic and bushy stunt viruses in saline lessens the destructive effect of heating on their precipitating power; with bushy stunt virus antiserum it also prevents the appearance of the phenomenon of inhibition. On the other hand, diluting the antisera in protein solutions (normal rabbit serum, rabbit serum albumin and pseudoglobulin, human serum albumin) enhances the destructive effect of heating on the precipitating power and with bushy stunt antiserum it also enhances the appearance of inhibition phenomenon. The inhibition is stronger after diluting in albumin solutions than after diluting in whole rabbit serum or in pseudoglobulin solution.

TABLE II.—*The Effect of Heating Tobacco Mosaic Virus Antiserum Diluted in Physiological Saline and in Normal Rabbit Serum.*

The antiserum was heated for 10 minutes at 75° C. and 80° C. at varying dilutions in saline and in normal rabbit serum diluted 1/10 in saline.

Dilution.	Diluent.	Temperature of heating.	Dilution of antisera.				
			1/50.	1/100.	1/200.	1/400.	1/800.
—	Saline	Unheated.	+	+	+	+	—
							o
1/10	,,	75° C.	+	+	+	—	—
						o	o
1/50	,,	,,	+	+	+	+	—
							o
1/50	Serum	,,	+	+	—	—	—
					o	o	o
1/100	Saline	,,	..	+	+	+	—
							o
1/100	Serum	,,	..	+	—	—	—
					o	o	o
1/10	Saline	80° C.	—	—	—	—	—
			o	o	o	o	o
1/50	,,	,,	+	+	—	—	—
					o	o	o
1/50	Serum	,,	—	—	—	—	—
			o	o	o	o	o
1/100	Saline	,,	..	+	—	—	—
					o	o	o
1/100	Serum	,,	..	—	—	—	—
				o	o	o	o

Symbols as in Table I.

TABLE III.—*The Effect of Heating Bushy Stunt Virus Antiserum Diluted in Physiological Saline and in Protein Solutions.*

The antiserum was heated for 10 minutes at 75° and 80° C. at varying dilutions in saline, in normal rabbit serum diluted 1/10 in saline and in 0·6 per cent. solutions in saline of rabbit serum albumin, pseudoglobulin or human serum albumin.

Dilution.	Diluent.	Temperature of heating.	Dilution of antisera. 1/50.	1/100.	1/200.	1/400.	1/800.
..	Saline	Unheated	+	+	+	+	−
							o
1/10	,,	75° C.	−	−	−	−	−
			i	o	o	o	o
1/50	,,	,,	+	−	−	−	−
				o	o	o	o
1/50	Rabbit serum	,,	−	−	−	−	−
			i	o	o	o	o
1/100	Saline	,,	..	+	−	−	−
					o	o	o
1/100	Rabbit serum	,,	..	−	−	−	−
				i	o	o	o
1/10	Saline	80° C.	−	−	−	−	−
			i	i	i	o	o
1/100	,,	,,	..	−	−	−	−
				o	o	o	o
1/100	Rabbit serum	,,	..	−	−	−	−
				i	i	o	o
1/100	Rabbit albumin	,,	..	−	−	−	−
				i	i	i	o
1/100	Rabbit pseudoglobulin	,,	..	−	−	−	−
				i	i	o	o
1/100	Human albumin	,,	..	−	−	−	−
				i	i	i	o

Symbols as in Table I.

Streng (1909) and earlier workers showed that diluting bacterial antisera in saline slows down the heat inactivation of agglutinins and also prevents the appearance of the inhibition phenomenon. Diluting in physiological saline is one of the means of preventing coagulation, i.e. the formation of large complexes of protein particles, which follows denaturation. Other means of preventing coagulation, such as varying pH or addition of substances like urea, etc., also reduce the rate at which heat causes antisera to lose their flocculating power (Marrack, 1938). These facts suggest that loss of the flocculating power of antibodies, or the exchange of this power for the ability to inhibit after heating, is a result of production of large complexes of protein particles. The fact that the addition of unspecific proteins before heating has an effect similar to using more concentrated antiserum solutions suggests that the complexes containing antibody are produced by the union of different protein fractions. They are formed during heating, for the addition of unspecific proteins after heating has no such effect. The possibility of unspecific proteins affecting the behaviour of antisera on heating was indicated by Pick (1902) (quoted by Streng, 1909), who showed that typhus agglutinins " purified" by salting out (i.e. when a considerable amount of unspecific protein, like

albumin, had been removed) were much less affected by heating than the original antiserum.

The experiments described below were made to test these conclusions and to gain additional information about the role played by unspecific proteins during heating of antisera.

The effect of heating euglobulin fractions of antisera separately and in the presence of other fractions.

The rate of destruction of flocculating power by heat is much slower in the case of euglobulin fractions of antisera than with either whole antisera or mixtures of euglobulin fractions and albumin.

Tables IV and V illustrate this for tobacco mosaic virus antibodies. The difference can be seen when the solutions are heated for varying lengths of time at 70° C. (Table IV), but it is much more definite when they are heated at 75° C. (Table V). The precipitation titre of the whole antiserum and of its euglobulin fraction heated in the presence of albumin falls from 1/640 and 1/160 respectively to less than 1/10 within 20 to 40 minutes, whereas the titre of euglobulin heated alone is almost unaffected after 40 minutes and is only reduced from 1/160 to 1/40 after 160 minutes.

TABLE IV.—*The Effect of Heating at 70° C. for Varying Lengths of Time Tobacco Mosaic Virus Antiserum and Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The antiserum was heated at a dilution 1/10 in saline. The euglobulin fraction of the antiserum (protein concentration 0·6 per cent.) was heated at a dilution of 1/10 in saline and in 0·4 per cent. rabbit albumin solution in saline.

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction.													
		1/80.	1/100.	1/120.	1/140.	1/160.	1/200.	1/240.	1/280.	1/320.	1/400.	1/480.	1/560.	1/640.	1/800.
Whole antiserum	0	+	..	..	..	+	..	..	..	+	..	..	..	+	—
	15	+	..	..	..	+	..	..	..	+	+	+	—	—	..
	30	+	..	..	..	+	..	..	..	+	+	—	—	—	..
	60	+	..	..	..	+	..	..	..	+	—	—	—	—	..
	120	+	..	..	..	+	+	+	+	—	..	..	..	—	..
	240	+	..	..	..	+	+	—	—	—	..	..	..	—	..
Euglobulin diluted in saline	0	+	+	+	+	—									
	15	+	+	+	+	—									
	30	+	+	+	—	—									
	60	+	+	+	—	—									
	120	+	+	+	—	—									
	240	+	+	—	—	—									
Euglobulin diluted in albumin.	0	+	+	+	+	—									
	15	+	+	+	—	—									
	30	+	+	—	—	—									
	60	+	+	—	—	—									
	120	+	—	—	—	—									
	240	+	—	—	—	—									

Symbols as in Table I. Only the results of precipitation are recorded. There was no inhibition.

TABLE V.—*The Effect of Heating at 75° C. for Varying Lengths of Time Tobacco Mosaic Virus Antiserum and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The solutions used for heating were as described in Table IV.

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction.							
		1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.
Whole antiserum	0	+	+	+	+	+	+	+	− o
	10	+	+	+	+	+	+	− o	− o
	20	− i	+	+	+	− o	− o	− o	− o
	40	− i	− o	− o	− o	− o	− o	− o	− o
	80	− i	− o	− o	− o	− o	− o	− o	− o
	160	− o	− o	− o	− o	− o	− o	− o	− o
Euglobulin diluted in saline	0	+	+	+	+	+	− o		
	10	+	+	+	+	+	− o		
	20	+	+	+	+	±	− o		
	40	+	+	+	+	±	− o		
	80	+	+	+	+	− o	− o		
	160	+	+	+	− o	− o	− o		
Euglobulin diluted in albumin	0	+	+	+	+	+	− o		
	10	+	+	+	− o	− o	− o		
	20	− i	− o	− o	− o	− o	− o		
	40	− i	− i	− o	− o	− o	− o		
	80	− i	− i	− o	− o	− o	− o		
	160	− i	− i	− i	− o	− o	− o		

Symbols as in Table I.

Table V shows inhibition as a result of heating tobacco mosaic virus antiserum. The effect is slight compared with that of bushy stunt virus (compare Tables I and VI) and it is only transient. In the mixture of euglobulin with albumin, however, the production of inhibition is much more definite and lasting, although still less than with bushy stunt virus. In the mixture the ratio albumin/globulin is 7/1, i.e. more than three times greater than in antiserum.

The presence of protein fractions other than euglobulin in the heated

solutions affects bushy stunt virus antibodies much more than tobacco mosaic virus antibodies. Table VI shows that heating at 70° C. is here sufficient to demonstrate considerable differences. The precipitating power of the euglobulin fraction of bushy stunt virus antiserum heated alone is but little affected

TABLE VI.—*The Effect of Heating at 70° C. for Varying Lengths of Time Bushy Stunt Virus Antiserum and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

The antiserum was heated at a dilution 1/10 in saline. The euglobulin fraction of the antiserum (protein concentration 0·75 per cent.) was heated at a dilution 1/10 in saline and in 0·4 per cent. rabbit albumin solution in saline.

Time of heating (in minutes).	Dilution of whole antiserum.								Dilution of the euglobulin fraction heated alone.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
0	+	+	+	+	+	+	+	—	+	+	+	+	+	+	—
								o							o
15	+	+	+	+	+	+	—	—	+	+	+	+	+	+	—
							o	o							o
30	+	+	+	+	+	—	—	—	+	+	+	+	+	+	—
						o	o	o							o
60	+	+	+	—	—	—	—	—	+	+	+	+	+	±	—
				i	o	o	o	o							o
120	+	+	—	—	—	—	—	—	+	+	+	+	+	±	—
			i	i	i	o	o	o							o
240	+	—	—	—	—	—	—	—	+	+	+	+	+	±	—
		i	i	i	i	o	o	o							o

Time of heating (in minutes).	Dilution of the euglobulin fraction heated in presence of rabbit serum albumin.														
	1/10.	1/12·5.	1/15.	1/17·5.	1/20.	1/25.	1/30.	1/35.	1/40.	1/80.	1/160.	1/200.	1/240.	1/280.	1/320.	1/400.
0	+	..	..	..	+	..	..	..	+	+	+	..	..	..	+	—
																o
15	+	..	..	..	+	..	..	..	+	+	+	+	+	—	—	..
														o	o	
30	+	..	..	..	+	..	..	..	+	+	+	—	—	—	—	..
												o	o	o	o	
60	+	..	..	..	+	+	+	—	—	—	—	..	..	..	—	..
								i	i	i	o				o	
120	+	+	+	—	—	..	..	..	—	—	—	..	..	..	—	..
				i	i				i	i	i				o	
240	+	—	—	—	—	..	..	..	—	—	—	..	..	..	—	..
		i	i	i	i				i	i	i				o	

Symbols as in Table I.

after 240 minutes, whereas the precipitation titre of the whole antiserum and of its euglobulin fraction heated in the presence of albumin falls from 1/640 and 1/320 respectively to 1/10. With the fall in the precipitation titre the phenomenon of inhibition appears. It first appears after about 60 minutes and its zone increases with the time of heating. It is definite before the precipitating power of heated solutions is totally destroyed. In such cases

precipitation occurs in more concentrated antibody solutions and inhibition in the more dilute solutions.

When bushy stunt virus antiserum is heated at 75° C., its precipitating power is completely destroyed within 10 minutes and is replaced by inhibition (Table I), and the euglobulin fraction of the antiserum heated with 0·4 per cent. of albumin behaves similarly. But when the euglobulin fraction is heated alone, its precipitating power is almost unaffected after 10 minutes, and is not much decreased after 160 minutes (Table VII). However, a zone of inhibition appears after 10 minutes of heating ; this looks like an antibody excess zone, and it narrows as the time of heating increases.

TABLE VII.—*The Effect of Heating at 75° C. for Varying Lengths of Time the Euglobulin Fraction of Bushy Stunt Virus Antiserum.*

The euglobulin fraction (protein concentration 0·75 per cent.) was heated at a dilution 1/10 in saline.

Time of heating.	Dilution of the euglobulin fraction.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
0	+	+	+	+	+	+	—
							o
10	—	—	+	+	+	+	—
	i	i					o
20	—	—	+	+	+	±	—
	i	i					o
40	—	+	+	+	+	—	—
	i					o	o
80	—	+	+	+	+	—	—
	i					o	o
160	+	+	+	+	+	—	—
						o	o

Symbols as in Table I.

Evidence has previously been given suggesting that normal rabbit serum proteins, while undergoing heat denaturation (in presence of 0·9 per cent. NaCl at pH near neutrality), combine with one another to form large mixed complexes (Kleczkowski, 1941). The results obtained with heated antisera suggest that antibodies can also unite with other proteins to form complexes. The serological behaviour of these complexes is determined by the other proteins present in the solution during heating and also by the type of antigen. When antibodies to either virus are heated with euglobulin fractions of the antisera only, they can still unite with and flocculate their antigens. That their properties are altered, however, is shown by slight changes in the precipitation. For example, tobacco mosaic virus precipitates are less nebulous and the floccules appear quicker than with unheated antibodies, and with bushy stunt virus antibodies the range of antibody excess inhibition is increased, so that precipitation is restricted to a narrower range of antigen/antibody ratios.

When antibodies are heated in the presence of protein fractions other than euglobulin much greater changes occur, and the differences between antigens of flagellar (tobacco mosaic) and somatic (bushy stunt) type become definite. The complexes formed in such mixtures of proteins can combine with their

antigens, but cannot cause flocculation, and their combination with antigen can prevent the latter from being precipitated subsequently by unchanged antibody. In a solution containing both changed and unchanged antibodies there is a competition between them, and the flocculation titre is the result of that competition. The titre, therefore, cannot be taken as a direct measure of the amount of antibody which remains unchanged, as authors who previously investigated the problem of the heat inactivation of antibodies have done. The result of the competition depends largely on properties of antigen particles. Consequently, after the same amount of heating in identical conditions tobacco mosaic virus antiserum can still precipitate its antigen, whereas bushy stunt virus antiserun has lost its precipitating power and inhibits. The results of the experiments described below show that such a competition does occur.

Effects of mixing heated and unheated antisera in varying proportions.

Antiserum to bushy stunt virus diluted 1/10 with saline was heated for 20 minutes at 80° C., so that its precipitating power was destroyed and inhibition was pronounced. The heated antiserum was then mixed with varying

TABLE VIII.—*The Effect of Mixing in Varying Proportions Heated and Unheated Bushy Stunt Virus Antiserum.*

The antiserum was heated for 20 minutes at 80° C. at a dilution of 1/10 in saline. This was mixed in varying proportions with unheated antiserum and the mixtures tested as described in Table I, or heated antiserum was first added to the solution of antigen and unheated antiserum followed after 1 hour.

Exp. number.	Ratio of heated antiserum to total amount of antiserum.	Dilution of the total antiserum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
A. *Heated and Unheated Antiserum added Simultaneously.*								
1	0·0	+	+	+	+	+	+	—
								o
2	0·2	+	+	+	+	+	—	—
							o	o
3	0·4	+	+	+	—	—	—	—
					o	o	o	o
4	0·5	+	+	—	—	—	—	—
				i	i	o	o	o
5	0·6	+	—	—	—	—	—	—
			i	i	i	o	o	o
6	0·66	—	—	—	—	—	—	—
		i	i	i	i	i	o	o
7	1·0	—	—	—	—	—	—	—
		ı	i	i	i	i	o	o
B. *Heated Antiserum Mixed first with Antigen Solution and Unheated Antiserum followed after 1 hour.*								
8	0·2	—	—	+	+	+	—	—
		i	i				o	o
9	0·4	—	—	—	—	—	—	—
		i	i	i	o	o	o	o

Symbols as in Table I.

amounts of unheated antiserum and the precipitating power of the mixtures was tested. The results are shown in Table VIII.

The competing action of changed antibodies is clearly shown. For example, the unchanged antiserum has a precipitation titre of 1/320, whereas a mixture of equal parts of heated and unheated antiserum has a titre of only 1/20, although there is sufficient unchanged antibody to give a titre of at least 1/160. Thus the competing action of the changed antibody has reduced the precipitation titre to one-eighth, showing the error made by taking the titre as a direct measure of the amount of unchanged antibody in the mixture.

Over the whole range of ratios in which heated and unheated antisera were mixed, there is a drop in titre due to the presence of changed antibody. This drop becomes greater with increasing amounts of heated antiserum in the mixture.

The results of heating bushy stunt virus antiserum for varying lengths of time at 70° C. agree closely with the results of mixing unheated and heated antiserum in varying proportions ; this can be seen by comparing Tables VI and VIII. With increasing time of heating at 70° C. (Table VI), as with increasing ratio of heated antiserum to the total amount of antiserum in the mixtures (Table VIII), the precipitation titre decreases. When the titre falls to about one-eighth of the original value the inhibition phenomenon appears, and the zone of inhibition widens with increasing time of heating or with increasing the ratio of heated to total antiserum. In both types of experiment inhibition occurs in the higher antiserum dilutions, while precipitation is still obtained in less dilute solutions. In less dilute solutions the concentration of unchanged antibody is still sufficient to cause precipitation, but in higher dilutions it is insufficient, and the competing action of changed antibody gives complete inhibition.

This similarity between the results shown in Tables VI and VIII suggests that during heating the ratio of changed to unchanged antibody increases, and that the fall in precipitation titre is an indication of this ratio.

Table VIII also shows that the addition of heated antiserum to antigen solution followed by unheated antiserum may give a result different from that when heated and unheated antisera are added simultaneously. Exps. Nos. 8 and 9 correspond to Nos. 2 and 3 respectively, except that in Nos. 8 and 9 unheated antiserum was added 1 hour after the heated antiserum, whereas in Nos. 2 and 3 heated and unheated antisera were added simultaneously. In some tubes the addition of the mixture gave precipitation, whereas there was inhibition when heated antiserum was added before the unheated. This difference again indicates competition between unchanged and changed antibodies. If enough changed antibody has already combined with antigen, unchanged antibody subsequently added cannot cause precipitation. However, if both kinds of antibody are added simultaneously, sufficient unchanged antibody may unite with antigen to give precipitation.

Similar experiments were made with tobacco mosaic virus antiserum and the results are shown in Table IX. Over the range of ratios of unheated to total antiserum from 0·0 to about 0·8 (Exps. Nos. 1–7) the precipitation titres are as they would be without any heated antiserum added, so that changed antibody present in heated antiserum has no visible influence on the precipitat-

TABLE IX.—*The Effect of Mixing in Varying Proportions Heated and Unheated Tobacco Mosaic Virus Antiserum.*

Methods as described for bushy stunt virus antiserum in Table VIII. Only the results of precipitation are recorded. There was no inhibition.

Exp. number.	Ratio of heated antiserum to total amount of antiserum.	Dilution of the total antiserum. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
A. *Heated and Unheated Antiserum Added Simultaneously.*								
1	0·0	+	+	+	+	+	+	−
2	0·2	+	+	+	+	+	+	−
3	0·4	+	+	+	+	+	−	−
4	0·5	+	+	+	+	+	−	−
5	0·6	+	+	+	+	+	−	−
6	0·7	+	+	+	+	−	−	−
7	0·8	+	+	+	−	−	−	−
8	0·9	−	−	−	−	−	−	−
B. *Heated Antiserum in the Exp. No. 8 Substituted by Equal Volume of Similarly Treated Normal Rabbit Serum.*								
9	0·9	+	+	−	−	−	−	−
C. *Heated Antiserum Mixed First with Antigen Solution and Unheated Antiserum followed after 1 Hour.*								
10	0·6	+	+	+	+	+	−	−
11	0·8	−	+	−	−	−	−	−

Methods of testing as in Table I.

ing action of unchanged antibody. However, when the ratio of heated to total antiserum is greater than 0·8 the effect of heat-changed antibody becomes apparent. In Exp. No. 8 there is no precipitation, although the amount of unchanged antibody is sufficient to give a titre of 1/20. This effect is due to the competing specific action of heated antiserum, for the presence of heated normal rabbit serum has no effect on precipitation (Exp. No. 9). Thus, unless heating is prolonged, for most purposes the titre of heated tobacco mosaic virus antiserum is a direct measure of the amount of unchanged antibody after heating.

Comparing Exps. Nos. 5 and 7 with Nos. 10 and 11 (Table IX) gives clearer evidence that heat-changed antibody can combine with tobacco mosaic virus without causing precipitation. The same amounts of heated and unheated antiserum were added to antigen in Exps. Nos. 5 and 10 and in Nos. 7 and 11, but in Nos. 5 and 7 the two were added as a mixture and in Nos. 10 and 11 the heated antiserum was added one hour before the unheated. As with bushy stunt virus, precipitation occurred in some tubes where heated and unheated antiserum were added at the same time, but not when heated antiserum was added first.

Inactivation of inhibiting power.

It has already been shown that heating for 10 minutes at 90° C. destroys

the inhibiting power of bushy stunt virus antiserum (Table I). Heating at lower temperatures for longer periods of time also does this. The inhibition titre at first increases, then remains approximately constant and later decreases. Thus at first there is a production of heat-changed antibodies, which have lost the precipitating power but still possess the ability to combine with antigen, while further heating produces a second change destroying all specific serological activity.

Behaviour of heated antibodies to other antigens.

To determine whether the results obtained with tobacco mosaic and bushy stunt virus antibodies apply at all generally, experiments were made with antibodies to potato virus " X," tobacco necrosis virus, pea nodule bacteria (*Rhizobium leguminosarum*) (317), human serum globulin and albumin.

Antisera to tobacco necrosis virus and potato virus " X " diluted 1/10 in saline were heated for 10 minutes at 80° C. Tobacco necrosis virus antiserum behaved like bushy stunt virus antiserum, the precipitation power being destroyed and replaced by inhibition. Potato virus " X " antiserum, on the other hand, behaved like tobacco mosaic virus antiserum ; its precipitation power was not destroyed, the only change being a decrease in the titre and an increased zone of antibody excess inhibition (cf. similar phenomenon with tobacco mosaic antiserum, Table V).

Tobacco necrosis virus, like bushy stunt virus, has quasi-spherical particles (Pirie *et al.*, 1938) and forms specific precipitate of the type " O," whereas potato virus " X," like tobacco mosaic virus, has anisodimensional particles (Bawden and Pirie, 1938*a*) and forms specific precipitates of the type " H." This indicates that the different results obtained with bushy stunt and tobacco mosaic virus antisera on heating are determined by their different shapes, and that the differences are probably generally applicable to all antigens of somatic (" O ") and flagellar (" H ") type. This is further supported by the fact that all the additional antigens tested give flocculation of the type " O," and their antisera on heating all behave very like bushy stunt virus antiserum.

Similar experiments were made with bacterial agglutinins. An antiserum to pea nodule bacteria diluted 1/10 in saline and also 0·1 per cent. solution of its euglobulin fraction in the presence and absence of other proteins were heated for 10 minutes at 80° C. The agglutinating power of the antiserum and of its euglobulin fraction heated in the presence of 0·4 per cent. rabbit albumin was destroyed and inhibition was definite, though less pronounced than with bushy stunt or tobacco necrosis viruses. On the other hand, the agglutination titre of euglobulin heated alone fell only from 1/3200 to 1/1600. The presence of 0·4 per cent. pseudoglobulin gave a rather different effect ; agglutination was destroyed, but there was no inhibition. When the euglobulin fraction of the antiserum and other fractions were mixed after being heated separately, agglutination occurred just as when the euglobulin was heated and tested separately.

Heating for 10 minutes at 90° C. destroyed both the ability to agglutinate and to inhibit.

TABLE X.—*The Effect of Heating at 70° C. for Varying Lengths of Time Antiserum to Pea Nodule Bacteria and the Euglobulin Fraction of the Antiserum in the Presence and Absence of Rabbit Serum Albumin.*

Preparation of antiserum.	Time of heating (in minutes).	Dilution of the antiserum or of the euglobulin fraction.							
		1/50.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	1/6400.
Whole antiserum diluted 1/10 in saline	0	+	+	+	+	+	+	+	−
	15	+	+	+	+	+	+	−	−
	30	+	+	+	+	+	+	−	−
	60	+	+	+	+	+	−	−	−
	120	+	+	+	+	+	−	−	−
	240	+	+	+	+	−	−	−	−
0·1 per cent. euglobulin solution in saline	0	+	+	+	+	+	+	−	−
	15	+	+	+	+	+	+	−	−
	30	+	+	+	+	+	+	−	−
	60	+	+	+	+	+	+	−	−
	120	+	+	+	+	+	+	−	−
	240	+	+	+	+	+	+	−	−
0·1 per cent. euglobulin solution in 0·4 per cent. albumin	0	+	+	+	+	+	+	−	−
	15	+	+	+	+	+	−	−	−
	30	+	+	+	+	−	−	−	−
	60	+	+	+	+	−	−	−	−
	120	+	+	+	−	−	−	−	−
	240	+	+	+	−	−	−	−	−

Table X shows the effect of heating the same preparations of bacterial antibodies for varying lengths of time at 70° C. There was no change in the euglobulin fraction heated alone up to four hours, whereas the presence of albumin caused a large fall in the titre ; a similar fall occurred when the whole antiserum was heated.

Antisera to both human serum albumin and human serum globulin diluted 1/10 in saline and 0·1 per cent. solutions of euglobulin fractions of the antisera in the presence and absence of 0·5 per cent. rabbit serum albumin were also heated for 15 minutes at 75° C. The precipitating power of whole antisera was destroyed and the phenomenon of inhibition well pronounced. Heating the euglobulin fractions of the antisera alone did not destroy their precipitating power, but when rabbit serum albumin was added to the euglobulin solutions before heating, the precipitating power was destroyed and the inhibition phenomenon was produced. Heating the albumin and the euglobulin separately and then mixing them had no such effect.

DISCUSSION.

Solutions of flocculating (precipitating or agglutinating) antibodies of rabbit antisera twice undergo changes during heating. The first change corresponds with an early stage of denaturation of serum proteins, and coincides with the appearance of active disulphide groups and with the formation of large complexes of changed protein particles (Kleczkowski, 1941). The ability to combine with antigen is not destroyed by this change. The second change corresponds with a further stage of denaturation and coincides with the loss of this ability.

The serological behaviour of antibody after the first change depends on the proteins present in the solution when antibodies are undergoing heat denaturation, for combination between heat-changed antibody particles and those of other proteins seems to occur and the other proteins largely determine the behaviour. Evidence for such a combination between pneumococcal antibody and other proteins has recently been provided by electrophoretic studies on heated horse antisera (Van der Scheer *et al.*, 1941 ; Krejci *et al.*, 1941).

The name " euglobulin complex " will be used for the complex formed when euglobulin fractions of antisera are heated alone. When other fractions are present, either when whole antisera are heated or when other proteins are added to the euglobulin, " mixed complexes," i.e. composed of particles of different fractions, are formed.

The formation of " euglobulin complexes " only slightly affects the flocculating power of antibody particles. The titre of antibody solutions to both flagellar and somatic types of antigen is affected similarly, and as compared with unheated antibody there are only slight differences in zones of flocculation and type of floccules. It is the formation of " mixed complexes " that is responsible for the wide differences known to exist between the behaviour of heated antisera to somatic and flagellar types of antigen. The " mixed complexes " can still combine with antigens but are unable to cause flocculation. This is true both for flagellar and somatic type of antigens. The difference between the two lies, not, as previously believed, in the difference between the heat stabilities of the antibodies, but in the different competitive effects of unchanged antibody and the " mixed complexes." The precipitating or agglutinating titre of a heated antiserum is a function of this competition, and this is governed by the type of antigen. With anisodimensional antigens, i.e. when the flocculation corresponds to the type " H," the competing action of " mixed complexes " is slight, and the titre of heated antiserum is an approximate measure of antibody which remains unchanged after heating. But with antigens like bushy stunt virus, tobacco necrosis virus, blood serum proteins and bacteria giving " O " type floccules, the " mixed complexes " interfere effectively with the flocculating action of unchanged antibodies and the titre is not a direct measure of the amount of these.

With " O " antigens there are two causes leading to a fall in flocculating titre of antisera after heating, the transformation of antibodies into a form unable to flocculate and the ability of the transformed antibodies to interfere with the flocculating action of unchanged antibodies. With " H " type of antigens, however, the fall in flocculating titre results almost entirely from the first cause. As most tests on heating antisera have been made with " O " type antigens, it is obvious that published work on rate of inactivation of antisera by heat (Streng, 1909 ; Madsen and Streng, 1910), in which agglutinating titres have been used as a direct measure of antibodies remaining unchanged, needs reconsidering. The use of flocculating titres for this purpose has been responsible for the belief that antibodies to different antigens differed in their resistance to heat, and led Marrack (1938) to suggest that " the destruction of some antibodies must be associated with earliest degrees of heat denaturation of proteins, while others can resist even complete denaturation." All the antibodies heated in this work behaved similarly, and the apparent differences

in the heat stability of the antisera resulted from differences in the competitive action of the " mixed complexes."

Why " mixed complexes " fail to flocculate the antigen, although they combine with it, is unknown. The amount and the properties of the material forming complexes with antibody particles during heating, however, probably determine this. If, like albumin, it readily forms stable suspensions, union with antigen is less likely to give an insoluble product than if, like heated globulin, it forms unstable suspensions. Similarly, the formation of complexes composed largely of serologically unspecific material can change the behaviour of proteins which function as antigens (Bawden and Kleczkowski, 1941).

The formation of serologically active antibody-complexes occurs only over a limited range of heating, further heating leading to a loss of all serological activity. This applies equally to both " O " and " H " type of antigens. This change is reflected in the drop of flocculating titre of heated euglobulin solutions and in the disappearance of inhibition in solutions of " mixed complexes." This stage probably corresponds with a further stage of protein denaturation, in which the antibody particles are so altered that they no longer contain specific groups capable of combining with antigen.

SUMMARY.

Experiments on the effect of heat on rabbit antisera to the following antigens have been made: human serum albumin and globulin, a strain of pea nodule bacteria (*Rhizobium leguminosarum*); and purified preparations of the following plant viruses—tobacco mosaic, potato " X," tomato bushy stunt and tobacco necrosis.

Antisera to the rod-shaped viruses (tobacco mosaic and potato " X ") behave like those to flagellar type antigens, whereas antisera to the other antigens named behave like those to somatic type antigens, much more heating being needed to destroy the flocculating power of the former. However, euglobulin fractions (containing antibodies) of all the antisera behave similarly, and they require more heat to destroy their flocculating power than do the original antisera.

Flocculating antibodies undergo at least two changes during heating. Complexes composed of antibody particles and of particles of other unspecific proteins present in the solution are first formed. Antibodies changed in this way can still combine specifically with antigens, but the result of this combination depends on the quantity and quality of unspecific proteins present in the solution during heating. Complexes formed when antibodies are heated in the presence of euglobulin fraction of the antiserum flocculate their antigens. Complexes formed when antibodies are heated in the presence of other serum fractions, notably albumin, cannot flocculate their antigens, although they combine with them; this combination interferes with the flocculating action of antibodies that are unchanged. The degree of this interference depends on the type of antigen, being large with antigens of type " O " and small with those of type " H." This fact, and not a difference in heat stability, explains the differences in the behaviour of heated antisera to the two types of antigen.

The second change of antibodies during heating corresponds with a further stage of denaturation and is shown by the loss of ability to combine with antigen.

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SOME PROPERTIES OF COMPLEXES FORMED WHEN ANTIGENS ARE HEATED IN THE PRESENCE OF SEROLOGICALLY UNSPECIFIC PROTEINS.

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KLECZKOWSKI (1941*b*, *c*) has presented evidence suggesting that rabbit antibodies can combine with other proteins while undergoing denaturation by heat. The serological behaviour of the complexes so formed depends on the protein with which the antibody has combined and on the type of antigen. Antibody-euglobulin complexes behave much like unchanged antibody, whereas antibody-albumin complexes can combine with, but not flocculate their antigens. Flagellar type antigens are much more readily flocculated by mixtures containing both unchanged antibody and antibody-albumin complexes than somatic type antigens. Because of this and not because of a greater resistance to heat, as previously believed, antisera to flagellar type antigens can be heated more than antisera to somatic type antigens without losing their ability to cause flocculation.

This paper shows that the serological behaviour of antigens after heating depends in the same way on what other proteins are present during heating and on whether the antigen is of the flagellar or somatic type. The antigens used were tomato bushy stunt and tobacco mosaic viruses and human serum