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albumins are still able to combine specifically with the antibodies. These products differ from the original albumins in that their precipitability by the antisera to the original albumins is greatly reduced. They do not differ from the original albumins in their diffusion rates. Precipitability of the products by the antisera can be restored by heating solutions of the products in the presence of salt.

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CONVERSION OF NON-PRECIPITATING AND INHIBITING PROTEIN COMPLEXES INTO FORMS AGAIN PRECIPITABLE BY THE ANTISERA TO THE ORIGINAL PROTEINS.

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WHEN certain pairs of proteins are heated in salt solutions, they combine during the initial stages of denaturation to form complexes. If one protein is present in the heated mixture in sufficient excess the resulting complex fails to precipitate with antiserum to the minor protein component, though it still combines specifically with its antibodies (Bawden and Kleczkowski, 1941*a*; Kleczkowski, 1941, 1943). This loss of precipitability with specific antiserum, resulting from combination of a protein with another protein into a complex, has now been found to be reversible, and this paper describes the reconversion of the non-precipitating complexes into precipitable forms by incubation with pepsin.

MATERIAL AND METHODS.

The proteins used were purified tomato bushy stunt virus, prepared as described by Bawden and Pirie (1943), and the albumin fractions of human, horse and rabbit serum, prepared as described in the preceding paper (Kleczkowski, 1945*a*). Commercial pepsin from the British Drug Houses, Ltd., was used. The same antisera to human and horse albumin were used as in the work described in the preceding paper. The antiserum to tomato bushy stunt virus was produced by intravenous injections into a

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rabbit of 2 ml. of 0·2 per cent. solution of the purified virus in 0·9 per cent. NaCl. Two injections a week were given over a period of three weeks. Serological precipitin tests were made as described in the preceding paper.

EXPERIMENTAL.

Complexes Formed Between Tomato Bushy Stunt Virus and Serum Albumins.

When a 0·05 per cent. solution of tomato bushy stunt virus in 1·0 per cent. NaCl at pH 7·0 is heated for 10 minutes at 80–82° C., it increases in opalescence and the virus undergoes partial denaturation; infectivity is lost, but the preparation still precipitates with virus antiserum and the protein is still resistant to the proteolytic activity of pepsin. When such a virus solution is heated similarly but in the presence of 1·0 per cent. human serum albumin there is less increase in opalescence, but the solution no longer precipitates with the virus antiserum, although it still precipitates with human albumin antiserum. The preparation still combines, however, with antibodies to the virus, for it specifically inhibits precipitation with virus subsequently added.

The ability of this solution to precipitate with the virus antiserum can be restored by incubation with pepsin. The changes in behaviour of the heated virus-albumin mixture during digestion with pepsin are shown in Table I. One volume of the mixture was added to four volumes of 0·03 per cent. pepsin in M/10 citric buffer at pH 2·6, and then incubated at room temperature. Samples were taken at intervals, neutralized, diluted to correspond to a virus concentration of 0·005 per cent. and tested against antisera to the virus and to human albumin.

Tests with the antiserum to the virus were made by adding 1 ml. of each sample to each of a series of tubes containing 1 ml. of the antiserum at varying dilutions. The test for inhibition was made after 3 hours' incubation at 50° C. by adding 0·1 ml. of 0·05 per cent. virus solution to the tubes in which there was no flocculation.

Tests with the antiserum to human albumin were made by adding 1 ml. of the antiserum at a constant dilution of 1/30 to a series of tubes each containing 1 ml. of the tested samples at varying dilutions. The test for inhibition was made by adding 0·1 ml. of 0·03 per cent. human albumin to the tubes in which there was no flocculation.

The samples were also examined for the amounts of material precipitable with 2 per cent. trichloroacetic acid. One volume of 10 per cent. CCl_3COOH was added to four volumes of each sample. The precipitate was centrifuged down, washed in 2 per cent. CCl_3COOH, and nitrogen was estimated by the micro-Kjeldahl method.

The first result of incubation with pepsin was that the heated virus-albumin mixture ceased to precipitate with human albumin antiserum and specifically inhibited the precipitation of human albumin. The reaction of the preparation with the virus antiserum then still remained unchanged. Further incubation destroyed the ability to inhibit precipitation of human albumin; with the loss of this power the preparation began to precipitate with the virus antiserum. At this stage little of the material in the incubated preparation was precipitated by 2 per cent. CCl_3COOH. Digestion was apparently complete after 48 hours' incubation, when only 6 per cent. of the original nitrogen remained precipitable with 2 per cent. CCl_3COOH. As the nitrogen content of the original virus was 4·5 per cent. of the total, all or almost all of the albumin had then been hydrolysed. Human albumin heated alone was hydrolysed by pepsin at the same rate as albumin heated with the virus.

Similarly, non-precipitating complexes formed by heating the virus with 20 times its amount of rabbit or horse serum albumin were restored to a state precipitating specifically with the virus antiserum. Again inhibition was replaced by precipitation

TABLE I.—*The Effect of Pepsin on the Complex Formed between Tomato Bushy Stunt Virus and Human Serum Albumin.*

Antigen.	Tests with the antiserum to the virus. Dilutions of the antiserum.						Tests with the antiserum to human albumin. Dilutions of the antigen.									Percentage of N precipitable with 2% CCl_3COOH.
	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	
0·005 per cent. unchanged bushy stunt virus	+++	+++	+++	+++	++	+	..	..	..	..	..	..	..	..	..	..
0·1 per cent. unchanged human albumin	..	..	..	..	..	..	−	−	+	+++	+++	++	+	+	−	..
Heated virus-albumin mixture	o	i	i	i	i	i	−	++	+++	+++	+++	++	+	−	−	100
Heated virus-albumin mixture incubated with pepsin. Neutralized after: 10 min.	o	i	i	i	i	i	i	i	i	i	o	o	o	o	o	91
Heated virus-albumin mixture incubated with pepsin. Neutralized after: 5 hours	o	o	i	i	i	i	i	o	o	o	o	o	o	o	o	58
Heated virus-albumin mixture incubated with pepsin. Neutralized after: 24 hours	++	+	o	i	i	i	o	o	o	o	o	o	o	o	o	21
Heated virus-albumin mixture incubated with pepsin. Neutralized after: 48 hours	+++	+++	+++	++	+	−	o	o	o	o	o	o	o	o	o	6
Heated virus-albumin mixture incubated with pepsin. Neutralized after: Control*	o	i	i	i	i	i	−	++	+++	+++	+++	++	+	−	−	100

* In the control the heated virus-albumin mixture was incubated at pH 2·6 without pepsin and neutralized after 48 hours.

+++ ++ + indicate the degree of precipitation.
− indicates the absence of precipitation if the test for inhibition was not made.
i indicates the presence of inhibition.
o indicates no precipitation and no inhibition.

as soon as most of the albumin component was hydrolysed beyond the stage of insolubility in 2 per cent. CCl_3COOH.

Incubation of virus-serum albumin complexes for 48 hours at pH 3·5 or at higher pH values, in conditions otherwise similar to those described above, did not result in any change in the reaction with the antiserum to the virus.

Complexes Formed Between Human and Horse Serum Albumin.

In complexes formed between bushy stunt virus and serum albumins one component resists peptic proteolysis whereas the other is hydrolyzed, but pepsin can also be used to convert non-precipitating complexes between two different albumins, both of which are susceptible to peptic proteolysis.

The complex formed between human and horse serum albumin which was not precipitable by the antiserum to human albumin was prepared by heating a solution containing 0·5 per cent. human and 2 per cent. horse serum albumin in 1 per cent. NaCl at pH 7·0 for 5 minutes at 80° C. To restore the ability of this solution to precipitate with the antiserum, the solution was incubated with pepsin at pH 3·5. For comparison a 2·5 per cent. solution of human albumin was heated in similar conditions and then similarly incubated with pepsin. One volume of each solution was added to two volumes of 0·033 per cent. pepsin in M/10 citric buffer at pH 3·5, and the mixtures incubated at room temperature. Samples were taken at intervals, neutralized, diluted to correspond to a 0·1 per cent. concentration of human albumin, and tested with the antisera to human and horse albumin. 1·0 ml. of each tested solution at varying dilutions was added to a series of tubes each containing 1·0 ml. of the antiserum to human albumin at a constant dilution of 1/30 or the antiserum to horse albumin at 1/35. The inhibition was tested by adding 0·1 ml. of 0·03 per cent. human albumin to the tubes where there was no precipitation with the antiserum to human albumin.

From the results shown in Table II it can be seen that after a few hours' incubation with pepsin the solution containing the complex became precipitable with the antiserum to human albumin, although it still remained precipitable with the antiserum to horse albumin. This was accompanied by a considerable decrease in opalescence, presumably due to disaggregation of the aggregates of heat denatured protein particles. With further incubation the precipitation zone with the antiserum to human albumin gradually widened in the direction of higher antigen concentrations, until eventually it corresponded with that given by human albumin which had been heated alone and similarly incubated with pepsin. From this it can be concluded that human albumin is hydrolyzed by pepsin at approximately the same rate irrespective of whether it is treated separately or is part of a complex.

It will also be seen from Table II that, as a result of incubation with pepsin, the zone of precipitation of the complex with the antiserum to horse albumin was shifted even more towards higher antigen concentrations than was the zone of precipitation with the antiserum to human albumin. This indicates that peptic proteolysis of the horse albumin component proceeded at a somewhat higher rate than that of the human albumin component.

A complex between horse and human albumin which did not precipitate with the antiserum to horse albumin was made by heating a solution containing 0·75 per cent. horse albumin and 1·5 per cent. human albumin in 1 per cent. NaCl for 10 minutes at 80° C. at pH 7·0. Peptic digestion also converted this solution into a form that precipitated with the antiserum to horse albumin. The solution was incubated with pepsin, then diluted to correspond to a 0·1 per cent. concentration of horse albumin,

TABLE II.—*The Effect of Pepsin on the Complex between Human and Horse Albumin Non-precipitable by Antiserum to Human Albumin.*

Antigen.	Tests with the antiserum to human albumin. Dilutions of the antigen: 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Tests with the antiserum to horse albumin. Dilutions of the antigen: 1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	1/1024.
Heated mixture of the two albumins	i	i	i	i	i	i	i	o	o	−	−	+	+++	+++	+++	++	+	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: ½ hour	i	i	i	i	i	i	i	o	o	..	..	..	..	..	..	..	..	..
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 1 ,,	i	i	o	o	o	i	i	o	o	..	..	..	..	..	..	..	..	..
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 3 hours	−	−	++	++	+	−	−	−	−	−	−	+	++	++	+	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 8 ,,	−	+	+++	+++	++	+	−	−	−	−	+	++	++	+	−	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 24 ,,	−	+++	+++	+++	++	+	−	−	−	+	++	++	+	−	−	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: 48 ,,	+	+++	+++	+++	++	+	−	−	−	++	++	+	−	−	−	−	−	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after: Control*	i	i	i	i	i	i	i	o	o	−	−	+	+++	+++	+++	++	+	−
Human albumin heated alone	−	−	+	+++	+++	+++	++	+	−									
Human albumin heated alone incubated with pepsin. Neutralized after: 3 hours	−	++	+++	+++	+++	++	+	−	−									
Human albumin heated alone incubated with pepsin. Neutralized after: 8 ,,	−	+++	+++	+++	++	+	−	−	−									
Human albumin heated alone incubated with pepsin. Neutralized after: 24 ,,	−	+++	+++	+++	++	+	−	−	−									
Human albumin heated alone incubated with pepsin. Neutralized after: 48 ,,	+	+++	+++	+++	++	+	−	−	−									
Human albumin heated alone incubated with pepsin. Neutralized after: Control*	−	−	+	+++	+++	+++	++	+	−									

* The controls were incubated without pepsin, otherwise in the same conditions. They were neutralized after 48 hours.

The symbols used as in Table I.

and tested with antisera to horse and human albumin as in the preceding experiment. The test for inhibition was made by adding 0·1 ml. of 0·03 per cent. horse albumin to those tubes where there was no precipitation with the antiserum to horse albumin. For comparison a solution containing 2·25 per cent. horse albumin but no human albumin was heated, incubated with pepsin and then tested similarly.

The results, given in Table III, show that after incubation with pepsin for 24 hours the solution containing the complex became precipitable with the antiserum to horse albumin; this precipitation, however, was restricted to a narrow zone of antigen dilutions, was weak and appeared slowly, so that the result was much less spectacular than that obtained in the preceding experiment, when human albumin was the minor component of the complex. This difference can probably be explained by the fact that horse albumin is somewhat more susceptible to peptic proteolysis than is human albumin. The precipitation zone of the complex, after it had been incubated with pepsin, with the antiserum to horse albumin coincided approximately with that given by horse albumin which had been heated alone, and the incubated with pepsin in similar conditions for the same length of time. This indicates that also horse albumin was hydrolyzed by pepsin at a comparable rate, whether it was treated separately or was a part of the complex. It can also be seen from Table III that the intensity of precipitation of the complex with the antiserum to human albumin was less affected by incubation with pepsin than that of horse albumin, which had been heated alone, with its antiserum, and there was less shift of the precipitation zone in the direction of higher antigen excess.

When complexes formed between human and horse albumin were incubated with pepsin for 24 hours at pH 3·5, the amount of material precipitable with 2 per cent. CCl_3COOH fell to about 75 per cent. of the original. After this incubation the complex was converted into a form precipitable with antiserum to either human or horse albumin. When either of the two albumins is incubated with pepsin at pH 2·6, a similar fall in the amount of material precipitable with 2 per cent. CCl_3COOH takes place within a few minutes and, simultaneously, the preparations cease to precipitate with the antisera to the original albumins used at the dilutions as in the experiments described above (compare the preceding paper, Kleczkowski, 1945*a*). This suggests that differences in pH and in substrate concentration not only influence the rate of peptic proteolysis, but also change the process qualitatively.

DISCUSSION.

If the inability of a protein complex to precipitate with the antiserum to its minor component is because the greater part of the complex does not participate in the serological reaction (Kleczkowski, 1945*b*), complete or partial disaggregation of the complex into its constituent parts would be expected to restore precipitability with the antiserum. This would only be expected, however, if the process causing disaggregation does not change the nature of the particles of the minor protein component in such a way that their ability to combine and precipitate with antibodies to the original antigen is destroyed. If pepsin is used to bring about such disaggregation, this condition is easily fulfilled when the minor component of the complex is resistant to peptic proteolysis, but the major component is susceptible. The complex formed between tomato bushy stunt virus and a serum albumin represents such a case. The albumin is hydrolyzed by pepsin in the same way as when treated separately, and the virus component is thus released in the form precipitable by the virus antiserum. In complexes formed between horse and human albumin, on the other hand, when both components are susceptible to peptic proteolysis, they are both hydrolyzed at

TABLE III.—*The Effect of Pepsin on the Complex between Horse and Human Albumin Non-precipitable by Antiserum to Horse Albumin.*

Antigen.		Tests with the antiserum to horse albumin. Dilutions of the antigen: 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	Tests with the antiserum to human albumin. Dilutions of the antigen: 1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.
Heated mixture of the two albumins		i	i	i	i	i	i	o	o	o	−	−	+	+++	+++	+++	++	+	−
Heated mixture of the two albumins incubated with pepsin. Neutralized after:	24 hours	+	++	+	−	−	−	−	−	−	+	+++	+++	+++	++	+	−	−	−
	48 ,,	++	+	−	−	−	−	−	−	−	++	+++	+++	++	+	−	−	−	−
	Control*	i	i	i	i	i	i	o	o	o	−	−	+	+++	+++	+++	++	+	−
Horse albumin heated alone		−	+	+++	+++	+++	++	+	−	−									
Horse albumin heated alone incubated with pepsin. Neutralized after:	24 hours	+	++	++	+	−	−	−	−	−									
	48 ,,	++	++	+	−	−	−	−	−	−									
	Control*	−	+	+++	+++	+++	++	+	−	−									

* The controls were incubated without pepsin, otherwise in the same conditions. They were neutralized after 48 hours.

The symbols used as in Table I.

approximately the same rate as when treated separately. The solution can be made precipitable by the antiserum to the minor component of the complex, however, by incubation with pepsin in such conditions that hydrolysis proceeds to a point where the complex is sufficiently disaggregated, but most of the protein particles of the minor component are not so changed as to be unable to precipitate with the antiserum to the original antigen.

In order to convert the virus-albumin complex into a form precipitable by the virus antiserum, most of the albumin component had to be hydrolyzed by pepsin at pH 2·6 beyond the stage of precipitability with 2 per cent. CCl_3COOH. A much smaller degree of peptic proteolysis carried out at pH around 3·5 was sufficient to convert the complexes formed between the two serum albumins into forms precipitable by the antisera to their minor protein components. Indeed, if this small extent of proteolysis were not sufficient to convert the complexes formed between the two albumins into such precipitable forms, the conversion would not be possible at all, because further proteolysis would destroy the ability of the components of the complexes to combine and to precipitate with the antibodies to the original albumins. There are indications that peptic proteolysis not only proceeds more slowly at pH around 3·5 than at pH 2·6, but that there are also qualitative differences in the process. It is possible that some links are preferentially broken at certain pH's.

The following reasons can be offered to account for the fact that in the virus-albumin complex much greater proteolysis of the albumin component was necessary to render it precipitable by the virus antiserum than that required to render a human albumin-horse albumin complex precipitable by the antiserum to its minor component. Firstly, in the virus-albumin complex the average number of serologically unspecific particles for one specific particle was much greater than in the human albumin-horse albumin complexes. Secondly, during proteolysis of the virus-albumin complex only the albumin component undergoes changes causing the release of the virus, whereas in the complex formed between the two serum albumins both components undergo changes tending to release each other.

There are a number of viruses with which no precipitin reaction has been obtained, even though they are known to be antigenic as they are neutralized specifically by antisera and some give positive complement-fixation test. No doubt this may often be a result of virus content in the tested solutions being too low to give a visible precipitate. However, it is also possible that some viruses fail to precipitate because they occur in the form of complexes with some unspecific material. If so, treatments that split the complex might release a serologically precipitable component. No example of this is known, but the possibility is emphasized by a recent work on tomato bushy stunt virus. As found in sap expressed from diseased plants this virus is precipitable by its antiserum, but when obtained from the fibre of infected tomato plants after passing through a fine-grinding mill, it is combined into a complex with a chromo-protein (Bawden and Pirie, 1944). This complex does not precipitate with the antiserum to the virus, although it combines specifically with the antibodies. By incubation with commercial trypsin the virus can be released from the complex in a form precipitable with the antiserum.

Non-precipitating complexes prepared by heating protein mixtures can produce antisera specific to only one of their components (Bawden and Kleczkowski, 1941*b*, 1942). In this way it is possible to produce antisera which, although they do not precipitate with the material used for their production, do precipitate with one of the components of the material. It is not certain whether the non-precipitating complexes as such cause production of the antisera, as non-precipitating solutions may contain some material not in the form of such complexes (Kleczkowski, 1945*b*); also, before

acting antigenically, the complexes may be broken *in vivo* into precipitable forms as they can be broken *in vitro* by means of pepsin or trypsin.

SUMMARY.

When tomato bushy stunt virus is heated together with a serum albumin, the virus can combine with the albumin to form a complex that does not precipitate with the antiserum to the virus. The virus, which is resistant to peptic proteolysis, can be recovered in the precipitable form by peptic hydrolysis of the complex. Non-precipitating complexes similarly formed between human and horse serum albumin can, in suitable conditions, be split by means of pepsin into forms again precipitable specifically by antisera, although both components of the complex are susceptible to peptic proteolysis.

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SPECIFIC PRECIPITATION OF ONE PROTEIN BY ANTISERUM TO ANOTHER.

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BRIEF mention has already been made of the fact that after tomato bushy stunt and tobacco mosaic viruses had been heated together with human serum albumin in suitable conditions, the viruses were precipitated by antiserum to the albumin (Bawden and Kleczkowski, 1941). This was interpreted as evidence that the viruses combine with the albumin during early stages of heat denaturation to form complex aggregates containing virus and albumin. However, only a small number of experiments was made, and the possibility of unspecific adsorption of one component during specific precipitation of the other was not definitely excluded. The present paper presents the results of more detailed experiments, showing that the precipitation is specific, and that the phenomenon is not peculiar to the two plant viruses. After heating together with other proteins, serum albumins and antibodies were specifically precipitated by antisera to the other proteins.

The material and methods of testing used in this work were the same as those used previously (Kleczkowski, 1945).

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