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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.

A. KLECZKOWSKI.*

From the Rothamsted Experimental Station, Harpenden, Herts.

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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).

* Beit Memorial Research Fellow.

EXPERIMENTAL.

The Serological Behaviour of Horse Albumin Heated in an Excess of Human or Rabbit Albumin.

Solutions containing the same amounts of albumins from horse, human and rabbit sera, all heated for 10 minutes at 80° C. in 1 per cent. NaCl solution at pH 7·0, were made by heating the proteins separately and in different combinations. The following scheme shows the details :

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated.

Solution No. 1 } mixed { (*a*) 1 ml. 1·5 per cent. horse alb. + 1 ml. 3 per cent. human alb.
(*b*) 2 ml. 1·5 per cent. rabbit alb.

Solution No. 2 } mixed { (*a*) 1 ml. 1·5 per cent. horse alb. + 1 ml. 3 per cent. rabbit alb.
(*b*) 2 ml. 1·5 per cent. human alb.

Solution No. 3 } mixed { (*a*) 2 ml. 0·75 per cent. horse alb.
(*b*) 2 ml. 1·5 per cent. human alb.
(*c*) 2 ml. 1·5 per cent. rabbit alb.

The total volume of each solution was made up to 12 ml. with 0·9 per cent. NaCl, so that each solution contained 0·125 per cent. of horse albumin, 0·25 per cent. human albumin and 0·25 per cent. rabbit albumin.

The three solutions were tested for their reactions with antisera to horse and human albumin. 1 ml. of each solution at varying dilutions was added to one series of tubes containing 1 ml. of horse albumin antiserum at 1/35, and to another series of tubes containing 1 ml. of human albumin antiserum at 1/15. The tubes were incubated for 3 hours at 50° C., and solutions which did not precipitate with antiserum to horse albumin were then tested for their ability to inhibit the precipitation of untreated horse albumin by adding 0·1 ml. of a 0·03 per cent. solution of horse albumin.

All three solutions precipitated with the antiserum to human albumin. After 3 hours at 50° C. the precipitates were removed by centrifugation, and 0·1 ml. of the antiserum to horse albumin diluted 1/3·5 was added to each supernatant fluid and incubation at 50° C. continued for another 3 hours. Solutions which did not then precipitate were tested for inhibition of precipitation of untreated horse albumin as described above. The results are given in Table I.

Comparing the behaviour of Solutions Nos. 1 and 2 with that of Solution No. 3, in Test A, it can be seen that when horse albumin had been heated *together* with human or rabbit albumin it was converted into a form which did not precipitate with the antiserum to horse albumin and which inhibited the precipitation of unchanged horse

TABLE I.—*Precipitation of Horse Albumin*

Antigen.	Weights of albumins used for preparation of the solutions (in mg.).			Test (A) with antiserum to horse albumin. Dilutions of antigen :							
	15.	30.	30.	1/1.	1/12.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
Solution No. 1 (horse + human)* + rabbit . .				i	i	i	i	i	i	i	o
,, No. 2 (horse + rabbit)* + human . .				i	i	i	i	i	i	i	o
,, No. 3 horse + human + rabbit . . .				−	−	+	+++	+++	++	+	−

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

albumin. It continued to precipitate after it had been heated alone and then mixed with the other two albumins which had been heated separately.

Precipitating Solutions Nos. 2 and 3 with antiserum to human albumin (Test B) had no effect on their subsequent behaviour when tested with antiserum to horse albumin (Test C), showing that nothing that reacts with the latter had been removed. The behaviour of Solution No. 1, containing horse and human albumin heated together, however, was strongly affected. In all those tubes in which precipitation occurred (except in the tubes where there was antigen excess), inhibition of precipitation of unchanged horse albumin with the antiserum to horse albumin disappeared and in one tube precipitation with the latter antiserum occurred. Clearly the precipitates produced by antiserum to human albumin had removed the material which reacts with the antiserum to horse albumin, and which causes inhibition of precipitation of unchanged horse albumin. Although Solution No. 1 at first did not precipitate with antiserum to horse albumin, it did so after absorption with antiserum to human albumin. This shows that all horse albumin was not converted into a non-precipitable and inhibiting form during the heating, but its precipitation was previously prevented by the presence of the inhibiting form.

The removal of horse albumin converted into the inhibiting form from solution No. 1 by the antiserum to human albumin is not caused by unspecific adsorption. This is suggested by the fact that Solution No. 2, which also contained horse albumin in the inhibiting form produced by heating it together with rabbit albumin, remained unaffected by specific precipitation of human albumin. Also horse albumin heated alone, which remained precipitable with the antiserum to horse albumin (Solution No. 3), remained unaffected by specific precipitation of human albumin. Thus only horse albumin that interacted with human albumin during heating the two *together* can be precipitated by the antiserum to human albumin.

The Serological Behaviour of Human Albumin Heated in an Excess of Horse or Rabbit Albumin.

The following experiment differed from the previous one in that human, instead of horse, albumin was converted into a non-precipitating and inhibiting antigen. This was done by heating the human albumin in the presence of an excess of horse or rabbit albumin ; to do this with human albumin a greater excess of horse or rabbit albumin is necessary than the excess of human or rabbit albumin sufficient to convert horse albumin into an inhibiting form. Except for the changes in proportions of various albumins, and in the combinations in which they had been heated, the experiment was carried out in the same way as the preceding one.

Three solutions, each containing 0·094 per cent. of human albumin, 0·375 per cent.

by Antiserum to Human Albumin.

Test (B) with antiserum to human albumin.								Test (C) of the supernatant fluids from Test (B) with antiserum to horse albumin.							
Dilutions of antigen :															
1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
−	−	+	+++	+++	+++	++	+	i	i	i	+++	o	o	o	o
−	−	++	+++	+++	+++	++	+	i	i	i	i	i	i	i	o
−	−	++	+++	+++	+++	++	+	−	−	+	+++	+++	++	+	−

* The brackets indicate that the materials were heated together.

horse albumin and 0·375 per cent. rabbit albumin, all heated for 10 minutes at 80° C. in 1 per cent. NaCl at pH 7·0, were prepared. The following scheme shows the differences between the solutions and details of their preparations:

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated:

Solution No. 4 } mixed { (*a*) 1 ml. 0·75 per cent. human alb. + 1 ml. 3 per cent. horse alb.
(*b*) 2 ml. 1·5 per cent. rabbit alb.

Solution No. 5 } mixed { (*a*) 1 ml. 0·75 per cent. human alb. + 1 ml. 3 per cent. rabbit alb.
(*b*) 2 ml. 1·5 per cent. horse alb.

Solution No. 6 } mixed { (*a*) 2 ml. 0·375 per cent. human alb.
(*b*) 2 ml. 1·5 per cent. horse alb.
(*c*) 2 ml. 1·5 per cent. rabbit alb.

The total volume of each solution was made up to 8 ml. with 0·9 per cent. NaCl.

The three solutions were tested with antisera to human and horse albumin as described above. The antiserum to human albumin was used at a constant dilution of 1/30, and the antiserum to horse albumin at 1/10. Tests for inhibition were made by adding 0·1 ml. of 0·03 per cent. human albumin to tubes where there was no precipitation with the antiserum to human albumin. All the solutions precipitated with the antiserum to horse albumin. The precipitates were removed by centrifugation, and 0·1 ml. of human albumin antiserum diluted 1/3 was added to each supernatant fluid. Tests for inhibition of precipitation of unchanged human albumin were made in tubes where there was no precipitation.

The results, given in Table II, show the same phenomena as those of the preceding experiment. Only in Solution No. 4, containing human albumin heated together with horse albumin, did precipitation with the antiserum to horse albumin produce changes in subsequent reaction with the antiserum to human albumin. The product of interaction between human and horse albumin in Solution No. 4, formed when the two were heated together, which inhibited precipitation of unchanged human albumin, was precipitated by the antiserum to horse albumin, except from the tubes where there was antigen excess in the reaction with this antiserum. A similar product of interaction between horse and rabbit albumin in Solution No. 5, formed when the two were heated together, was unaffected by precipitation of horse albumin by its antiserum from the same solution; neither was human albumin in Solution No. 6 precipitable by its antiserum after it had been heated alone and then mixed with horse and rabbit albumin heated separately.

It can also be seen that in three tubes containing Solution No. 4, from which precipitates formed with the antiserum to horse albumin had been removed, there was

TABLE II.—*Precipitation of Human Serum*

Antigen.	Weights of albumins used for preparation of the solutions (in mg.)	Test (A) with antiserum to human albumin. Dilutions of antigen:								
	7·5. 30. 30.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/25
Solution No. 4 . (human + horse)* + rabbit .		i	i	i	i	i	i	i	i	o
„ No. 5 . (human + rabbit)* + horse .		i	i	i	i	i	i	i	i	o
„ No 6 . human + horse + rabbit .		−	−	+	+++	+++	+++	++	+	−

precipitation with the antiserum to human albumin. This indicates that this solution contained some of human albumin which did not interact with horse albumin to give the product non-precipitable by the antiserum to human albumin.

The Serological Behaviour of Rabbit Antibodies Heated in an Excess of Human or Horse Albumin.

Similar experiments were made with antibodies using "euglobulin" fraction of the antiserum to tomato bushy stunt virus. This fraction was prepared by adding one volume of a saturated ammonium sulphate solution to two volumes of the antiserum. The resulting precipitate was centrifuged down, washed in 1/3 saturated $(NH_4)_2SO_4$, redissolved in a volume of water corresponding to 3/4 volumes of the original antiserum, dialysed for 24 hours against 0·9 per cent. NaCl, and the total volume was adjusted to that of the original antiserum by adding 0·9 per cent. NaCl. The protein concentration of the solution was 1·0 per cent., and it contained most of the precipitating antibodies of the antiserum.

Three solutions each containing 0·033 per cent. of the "euglobulin" fraction of the antiserum to the virus, 0·166 per cent. of human albumin and 0·166 per cent. of horse albumin, all heated for 10 minutes at 75° C. in 0·9 per cent. NaCl at pH 7·0, were prepared as shown in the following scheme:

The fluids *a*, *b* and *c* were heated separately, then cooled and mixed as indicated:

Solution No. 7 } mixed { (*a*) 1 ml. 0·2 per cent. euglob. + 1 ml. 1 per cent. human alb.
(*b*) 2 ml. 0·5 per cent. horse alb.

Solution No. 8 } mixed { (*a*) 1 ml. 0·2 per cent. euglob. + 1 ml. 1 per cent. horse alb.
(*b*) 2 ml. 0·5 per cent. human alb.

Solution No. 9 } mixed { (*a*) 2 ml. 0·1 per cent. euglob.
(*b*) 2 ml. 0·5 per cent. human alb.
(*c*) 2 ml. 0·5 per cent. horse alb.

The volume of each solution was made up to 6 ml. with 0·9 per cent. NaCl.

The effect of treatment of the three solutions with antisera to human and horse albumin was tested. Three 1·5 ml. samples of each solution were measured separately into nine tubes. 1·7 ml. of undiluted antiserum to human albumin was added to one sample of each solution, 1·4 ml. of undiluted antiserum to horse albumin and 0·3 ml. 0·9 per cent. NaCl were added to the second sample, and 1·7 ml. of 0·9 per cent. NaCl to the third. (The amounts of the antisera corresponded with constant antiserum optimal proportions with human or horse albumin, respectively, present in

Albumin by Antiserum to Horse Serum Albumin.

Test (B) with antiserum to horse albumin.									Test (C) of the supernatant fluids from Test (B) with antiserum to human albumin.								
Dilutions of antigen:																	
1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1 64.	1/128.	1/256
+	++	+++	+++	+++	+++	++	+	±	.	i	i	+++	+++	++	o	o	o	o
+	++	+++	+++	+++	+++	++	+	±	.	i	i	i	i	i	i	i	i	o
+	++	+++	+++	+++	+++	++	+	±	.	−	−	+	+++	+++	++	+	−	−

Symbols as in Table I.

* The brackets indicate that the materials were heated together.

TABLE III.—*Precipitation of Complexes Formed by Antibodies to Tomato Bushy Stunt Virus with Human and Horse Albumin.*

Test number.	Solution of antibodies to bushy stunt virus.	Weight of materials used for preparation of the solutions (in mg.). 2.	10.	10. (Albumin.)	Treatment.	Dilutions of the solutions of antibodies. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.
1	Solution No. 7	(euglob. + human)* + horse			Incubated with 0·9 per cent. NaCl (control)	i	i	i	i	o	o
2	,, No. 8	(euglob. + horse)* + human				i	i	i	i	o	o
3	,, No. 9	euglob. + human + horse				+++	+++	+++	++	+	−
4	,, No. 7	(euglob. + human)* + horse			Absorbed with antiserum to human albumin	++	+	+	o	o	o
5	,, No. 8	(euglob. + horse)* + human				i	i	i	i	o	o
6	,, No. 9	euglob. + human + horse				+++	+++	+++	++	+	−
7	,, No. 7	(euglob. + human)* + horse			Absorbed with antiserum to horse albumin	i	i	i	i	o	o
8	,, No. 8	(euglob. + horse)* + human				+++	++	+	o	o	o
9	,, No. 9	euglob. + human + horse				+++	+++	+++	++	+	−

* The brackets indicate that the materials were heated together.

The symbols used as in Table I.

1·5 ml. of each sample.) The mixtures were incubated for 1 hour at 37° C., and then left overnight at 2° C. Precipitates formed in the mixtures which contained the antisera were then removed by centrifugation. Nine different fluids were thus obtained.

Each fluid was then tested for precipitation with the virus and for inhibition of its precipitation by unchanged antiserum to the virus. 1 ml. of varying dilutions of each fluid was added to a series of tubes, each containing 1 ml. of a 0·005 per cent. solution of the virus in 0·9 per cent. NaCl. Inhibition of precipitation of the virus by unchanged virus antiserum was tested by adding 0·1 ml. of the antiserum at a dilution of 1/25 to the tubes where there was no flocculation.

From the results, given in Table III, it can be seen that when the " euglobulin " fraction of the antiserum to the virus had been heated *together* with either human or horse albumin (Solutions Nos. 7 and 8 respectively), antibodies were converted into a form which did not precipitate with the virus, although they combined with it and inhibited its precipitation with unchanged antiserum (Tests Nos. 1 and 2). The antibodies were not converted into such form and were still able to precipitate with the virus when the " euglobulin " fraction was heated alone, and then mixed with human and horse albumin heated separately (Solution No. 9, Test No. 3).

Antibodies converted into the non-precipitating and inhibiting form by heating together with a serum albumin could be precipitated by the antiserum to this particular albumin, but they were not affected by precipitation of another albumin from the same solution. This is shown by the fact that the product causing inhibition of precipitation of the virus could be removed from Solution No. 7, but not from Solution No. 8 by human albumin antiserum (Tests Nos. 4 and 5), and from Solution No. 8 but not from Solution No. 7 by horse albumin antiserum (Tests Nos. 8 and 7). This seems to exclude the possibility that antibodies converted into the non-precipitating form are liable to unspecific adsorption during specific precipitation of a serum albumin. Also antibodies which were not heated together with either of the two albumins, and which were, therefore, able to precipitate with the virus (Solution No. 9), remained unaffected by the precipitation of either albumin by its antiserum (Tests Nos. 6 and 9).

After removal of the inhibiting material both solutions precipitated the virus, although they did so at a lower titre than that given by Solution No. 9, which contained the " euglobulin " fraction heated separately (Tests Nos. 3, 6 and 9). This seems to show that both Solutions Nos. 7 and 8 contained some antibodies which did not interact with the albumins to form products non-precipitable by the virus. The presence of such precipitating antibodies in heated antisera to tomato bushy stunt virus was shown by Bawden and Kleczkowski (1942*b*) by the separation of precipitating fractions from non-precipitating and inhibiting antisera by means of $(HN_4)_2SO_4$ or centrifugation.

DISCUSSION.

Evidence has previously been given that when mixtures of different proteins are heated, heat denatured particles of different kinds of protein can combine to form complex aggregates. For example, when mixtures of certain proteins in salt solutions undergo heat denaturation, interaction products are formed with certain properties different from those of each separate component. Such products are formed at the expense of both protein constituents, and can differ from them in their solubility and precipitability with salts (Kleczkowski, 1941*a*, 1943), in electrophoretic mobility (Van der Scheer *et al.*, 1941), or in serological reactions with specific antigens or antibodies (Kleczkowski, 1941*b* ; Bawden and Kleczkowski, 1941, 1942*a* and *b*). Further evidence for the complex formation is given by the effect of salts on the formation of the interaction products, and by the effect of pepsin on some of such products. The

presence of salts in heated protein mixtures promotes the formation of the interaction products, and the efficiency of different salts in promoting their formation follows Hardy's law, as does their efficiency in causing aggregation of heat denatured protein particles eventually leading to flocculation (Kleczkowski, 1943). If an interaction product is formed by two proteins, one of which is resistant to peptic proteolysis and the other susceptible, certain serological properties of the resistant component can be restored when the other component is hydrolysed by pepsin (Kleczkowski, 1945).

The fact, demonstrated in this work, that a protein heated in the presence of another protein can be specifically precipitated by the antiserum to the other protein, gives the most convincing evidence for the formation of complex aggregates. No alternative explanation of the phenomenon seems to be possible. The serological behaviour of such complex aggregates is most simply explained by assuming that they contain a number of particles of both proteins, and that the ratio of the numbers in an average complex aggregate depends on the ratio of the amounts of the proteins in the heated mixture. The failure of such a complex, composed of two components in unequal quantities, to precipitate with the antiserum to the minor component in spite of combination with the antibodies, is simply explained if we assume that precipitation of antigens by antibodies depends on mutual blocking of the groups determining solubility. As the major component of the complex consists of material which does not participate in the reaction with the antibodies specific for the minor component, only a small proportion of all hydrophilic groups is blocked and the whole complex remains in solution, although its minor component is combined with antibodies. As would be expected, the complex is precipitable by the antiserum to its major component.

A similar interpretation would explain the inability of complexes formed between antibodies and excess serum albumin to precipitate with their specific antigens. Combination of the antibody with its specific antigen would leave hydrophilic groups of the albumin unaffected, and consequently precipitation of the whole complex does not take place. Such antibody-albumin complex is precipitable by the antibodies to the albumin because the albumin is in excess.

As antibody or antigen particles present in such complexes combine with their respective antigens or antibodies, it follows that some at least of their specifically combining groups still remain accessible.

Solutions containing complexes non-precipitable by the antigens or antibodies specific for their minor components also contain certain amounts of precipitable material. This can be demonstrated either by removing the complex, by precipitation with the antiserum specific to its major component, or by fractional precipitation with $(NH_4)_2SO_4$ or by centrifugation (Bawden and Kleczkowski, 1942*b*). In the case of antibodies to " H "-type antigens, e.g. tobacco mosaic virus, the inhibiting activity of the antibody-albumin complex is so small that the precipitating activity of the antibodies which remained precipitable is demonstrable in the presence of the complex (Kleczkowski, 1941*b*).

The presence of serologically precipitable material in the preparations of non-precipitable complexes shows that not all protein particles form similar complex aggregates with particles of another protein. Several explanations of this, not mutually exclusive, can be offered. Firstly some of the protein may remain unaffected by heating. Secondly some protein particles may combine with the particles of the same kind to form aggregates similar to those formed when the protein is heated alone. Thirdly complex aggregates may be formed with ratios of constituents distributed around an average value. In some complex aggregates the ratio of a number of particles of the minor protein component of the heated mixture to that of the major component may thus be sufficiently high to allow their precipitation with antiserum

or antigen specific to the minor component, and to prevent it with antiserum to the major component.

SUMMARY.

Products of interaction between human and horse albumin, formed during heat denaturation of mixtures of the two, could be precipitated by the antisera to either albumin. The product formed in mixtures, where human albumin was in excess, combined with antibodies to horse albumin, but did not precipitate with them. It precipitated with antibodies to human albumin. The converse of this was true when horse albumin was in excess over human albumin in heated mixtures.

Products of interaction formed between antibodies to bushy stunt virus and human or horse serum albumin, formed when mixtures were heated, could combine, but not precipitate, with the virus, although they could be specifically precipitated with antiserum to the albumin.

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LAEVULOSE TOLERANCE IN ACUTE HEPATITIS.

C. H. GRAY.

From the E.M.S. Sector 9 Biochemical Laboratory, Manor Hospital, Epsom, and the Biochemical Department, King's College Hospital, London.

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THIS paper describes certain changes in laevulose tolerance which occur during acute hepatitis. It will be shown that the marked decrease in laevulose tolerance which occurs in many cases of acute hepatitis is probably present in the early stages of all cases of the disease, that the laevulose tolerance curve alters its shape during the progress of the condition, and in most cases becomes normal during the early stages of recovery. Moreover, marked impairment of laevulose tolerance can occur where hyperbilirubinaemia is minimal and clinical jaundice absent. Evidence is presented that such cases of acute hepatitis without jaundice are not necessarily to be regarded as mild cases of the disease. In fact, intensity of jaundice *per se* is no indication of the severity of the underlying liver lesion. It is suggested that impairment of laevulose tolerance may be greatest during the prodromal stages of the disease. The practical application of these findings is discussed in relation to the routine investigation of such cases.

METHODS.

In all the later cases, serum bilirubin was measured either by the method of Malloy and Evelyn (1937), or by a photoelectric modification of the method of Rappaport