4. With acetone-dried deficient cell preparations, pyridoxal in the presence of ATP is not a satisfactory standard. This is probably due to the low activity of the pyridoxal-phosphorylating system in such preparations.

5. Sulphate, phthalate, phosphate and pyrophosphate inhibit the decarboxylation of tyrosine by acetone-dried deficient cells in the presence of small amounts of codecarboxylase; the inhibition by sulphate is reduced or abolished by an increase in the amount of codecarboxylase added. These substances do not inhibit the decarboxylation of tyrosine by intact deficient cells in the presence of pyridoxal; with acetone-dried preparations of cells grown in a medium containing excess pyridoxal they have little or no inhibitory effect. The action of these salts is, therefore, interpreted not as an inhibition of the holoenzyme, but as an interference with the reaction between codecarboxylase and the apoenzyme.

6. Vitamin $B_6$-free intact cells of the organism do not decarboxylate tyrosine in the presence of either synthetic or natural codecarboxylase.

I am indebted to Prof. I. C. Gunsalus for a subculture of *Strep. faecalis* R (A.T.C.C. 8043), to Dr K. Folkers of Merck and Co. Inc. U.S.A., for gifts of pyridoxal and calcium pyridoxal-5-phosphate, to Messrs Boots Pure Drug Co. Ltd. for a gift of ATP (barium salt), and to Messrs Roche Products Ltd. for gifts of pyridoxalacetal-3-phosphate. I wish to thank Prof. J. H. Burn, F.R.S., for the hospitality of this Department; I am grateful to Drs H. Blaschko and D. D. Woods for much help and advice, and to the Medical Research Council for a studentship.

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# A Quantitative Study of Complex Formation in Heated Protein Mixtures

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When proteins are heated together under certain conditions, the heat-denatured particles of different proteins can combine to form complex aggregates. Previous work on such complexes has been solely qualitative. It has shown that complexes are formed (Kleczkowski, 1945) and that they can differ from their components in solubility and precipitability by salts (Kleczkowski, 1941*a*), electrophoretic mobility (Van der Scheer, Wyckoff & Clarke, 1941), isoelectric point (Kleczkowski, 1946) and serological behaviour (Kleczkowski, 1941*b*; Bawden & Kleczkowski, 1941, 1942*a*, *b*), but there has been no quantitative work on their formation or properties. The present paper describes (i) the effect of varying the concentrations and ratios of the components in heated mixtures on the constitution of the resulting complexes, and (ii), the effect of variations in the constitution of the complexes on their solubility and serological behaviour.

## MATERIALS AND METHODS

The proteins used were tobacco mosaic virus (TMV), tomato bushy stunt virus (BSV) and human-serum albumin. The viruses were purified by the methods described by Bawden & Pirie (1943). For convenience the heat-denatured viruses will be referred to as viruses, although they are non-infective and heat-denatured TMV has also lost its serological activity and its nucleic acid. The albumin was a crude unfractionated preparation from human serum. After removal of the globulin by half saturation with $(NH_4)_2SO_4$ and filtration, the filtrate was saturated with the salt. The precipitated albumin was collected by filtration, dissolved in water and dialyzed against water. Toluene was added as an antiseptic.

In all experiments designed to test the effect of heat, the protein solutions were made with M/15 phosphate buffer at pH 6·8 and heated in thin-walled test tubes immersed in a water bath. Except when otherwise stated, the time of heating was 10 min. and the temperature of the water bath 83°. Under these conditions, when heated alone, both viruses

coagulate, whereas when they are heated in the presence of sufficient albumin, no coagulum separates.

The complexes formed between albumin and each of the viruses were isolated by precipitation with $(NH_4)_2SO_4$, which was used at concentrations insufficient to precipitate albumin after this protein had been heated alone. The precipitates were sedimented by centrifuging for 10 min. at 6000 rev./min. and washed 3 times with 5 % (w/v) trichloroacetic acid. Their N, P and carbohydrate contents were estimated, and the relative colour intensity given by them in the May & Rose (1922) test for tryptophan was determined. From these results the total protein and the ratio of the constituent proteins in the complexes were obtained. N was estimated by the micro-Kjeldahl method, and translated into protein by multiplication by 6·4. P and carbohydrate were estimated colorimetrically (Kleczkowski, 1946). The colorimetric test for tryptophan differed in some respects from Bates's (1937) modification of the May & Rose (1922) test. The reagent (6 ml.) was added to 0·4 ml. of a protein solution or suspension, the mixture incubated for 5 min. at 75° and then cooled; the intensities of the blue colours formed by different protein preparations were compared in a colorimeter of Duboscq type. (The reagent consisted of 25 ml. of conc. HCl + 0·5 ml. of 5 % (w/v) *p*-dimethylaminobenzaldehyde in 10 % (w/v) $H_2SO_4$ + 0·2 ml. of 1 % (w/v) $NaNO_3$. The constituents were freshly mixed for each test.)

## RESULTS

### *Aggregation of the albumin heated alone*

As a preliminary to using precipitation with ammonium sulphate for isolating the complexes, the effect of heating on the albumin alone was studied. It was found that the precipitability of heated albumin by the salt depends on several factors such as the temperature and the duration of heating, the concentration of albumin during heating, the concentration of albumin after heating when the salt is added, and the lapse of time between adding the salt and centrifuging. For the purpose of this work variation in the albumin concentration during heating was the most important of these factors, and its effect was studied in detail. Fig. 1 shows the effects of heating 1, 0·5 and 0·25 % albumin solutions. After cooling, the 1 and 0·5 % albumin solutions were diluted to four times and twice their volumes respectively with M/15 phosphate buffer at pH 6·8 to bring all the solutions to the same protein concentration. Different amounts of saturated ammonium sulphate solution were added to different samples from each solution. The mixtures were kept for 15 hr. at room temperature, centrifuged for 10 min. at 6000 rev./min. and the amounts of sedimented protein were estimated. These, expressed as percentages of total albumin, are plotted in Fig. 1 against concentrations of ammonium sulphate, expressed as percentages of saturation.

It will be seen that the higher the concentration of the albumin during heating, the lower was the minimum concentration of the salt necessary to produce precipitation and the greater was the amount precipitable by salt concentrations between 31 and 50 % saturation. On the other hand, at 60 % saturation, which precipitated only about 4 % of the unheated albumin, about 85 % of the total albumin was precipitated from all three heated solutions. It seems that the proportion of albumin denatured by heat did not depend appreciably on the albumin concentration during heating. The 85 % of the total albumin which was rendered precipitable by 60 % saturated ammonium sulphate probably consisted of fractions that denature rapidly at 83°, and the remaining 15 % (probably mainly glycoprotein) of fractions with lower denaturation rates or with solubilities not greatly affected by heat.

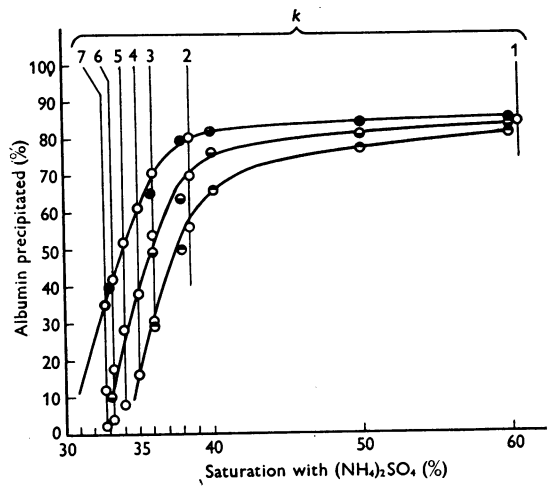


Fig. 1. Percentages of heated albumin precipitated by different concentrations of ammonium sulphate. ●, Albumin heated in 1 % solution; ◒, in 0·5 % solution; ◓, in 0·25 % solution; ○, values of $0·85p$ ($p$ being obtained from equation (1); for significance of $k$ see text).

Pedersen (1931) has shown that when albumin solutions, ultracentrifugally homogeneous before heating, are heated under conditions in which coagulation does not occur, they contain after heating, components with different sedimentation constants. From theoretical considerations of the kinetics of aggregation it is to be expected that the higher the concentration of a heated albumin solution, the greater will be the extent of aggregation. It seems likely that precipitability with ammonium sulphate at concentrations lower than 60 %, at which all the denatured albumin separates, depends on the extent to which the denatured protein molecules have aggregated. Supporting evidence for this assumption was obtained from the results of an experiment in which the three heated albumin solutions were adjusted to a concentration

of 0·25% and centrifuged for 30 min. at 40,000 rev./min. in an air-driven angle centrifuge. The pellets produced contained 60, 40 and 20% of the total protein from the solutions heated at 1, 0·5 and 0·25% albumin respectively, whereas no pellet was obtained from an unheated solution of the albumin. These proportions corresponded approximately with those of the amounts precipitated from the three solutions by 35% saturated ammonium sulphate (Fig. 1).

From Smoluchowski's (1916, 1918) theory of aggregation in monodisperse colloidal solutions with approximately spherical particles, the formula (1) is derived for the proportion ($p$) of material composed of $k$ and more primary particles;

$$p = \sum_{i=k}^{\infty} \frac{i\alpha^{i-1}}{(1+\alpha)^{i+1}} = \left(\frac{\alpha}{1+\alpha}\right)^{k}\left(\frac{k}{\alpha}+1\right), \qquad (1)$$

where $\alpha = 8\epsilon\pi RDv_0 t$, and $R$ is the radius of the primary particle (in cm.), $D$ is its diffusion constant (in cm.$^2$/sec.), $v_0$ is the original number of primary particles in unit volume (ml.), $t$ is the length of time of aggregation (in sec.) and $\epsilon$ is the fraction of collisions resulting in permanent combination.

The results shown in Fig. 1 fit formula (1) if three assumptions are made: first, that the 85% of albumin denatured rapidly, so that during most of the 10 min. heating at 83° only aggregation was occurring; secondly, that all the heat-denatured albumin molecules were of approximately the same size and equally likely to aggregate; thirdly, that the amounts of albumin precipitated from the three heated solutions by a given concentration of ammonium sulphate were at least roughly equal to the amounts of albumin which existed in the form of aggregates composed of at least $k$ primary particles. Different values of $k$ would then correspond with different concentrations of the salt. This can be assumed in spite of the fact that, whereas $k$ takes only integral values, the amount of precipitated albumin is a continuous function of the concentration of ammonium sulphate.

The value of $\epsilon$ will be expected to be low because heat-denatured albumin molecules remain charged at pH 6·8. All the values determining $\alpha$, except $v_0$, are constant, so that the value of $\alpha$ is proportional to the concentration of heated albumin solutions.

The value of $\alpha$ for one albumin concentration was so chosen that when, for one chosen value of $k$, the value of $p$ was fitted to the curve in Fig. 1, the value of $p$ for another albumin concentration fitted to the corresponding point of the other curve. Thus the values taken for $\alpha$ were 0·7, 1·4 and 2·8 for 0·25, 0·5 and 1% albumin solutions respectively. It will be seen from Fig. 1 that when the values of $p$, corresponding to different values of $k$, were made to fit to the curve for one of the albumin concentrations, the other two values fitted corresponding points on the other two curves through the whole range.

From the results of fitting the formula (1) to the curves in Fig. 1 it can be concluded that only about half of the material was in the form of aggregates composed of at least five, three and two primary particles in the heated solutions containing 1, 0·5 and 0·25% albumin respectively, and in the 0·25% albumin solution the proportion of material in aggregates containing seven or more primary particles was negligible. The aggregation did not, therefore, progress very far. It can also be concluded that, because the value of $\epsilon$ is of the order of $10^{-8}$, only one out of about $10^8$ collisions resulted in permanent combination. (There are reasons to suppose that the value of $\epsilon$ decreases rapidly with decreasing temperature.) So many assumptions are involved, however, that these conclusions can only be considered as tentative.

### *Estimations of the ratios of the constituents in the complexes*

Three solutions were heated: *A*, 0·042% TMV and 0·25% albumin; *B*, 0·042% BSV and 0·25% albumin; *C*, 0·25% albumin. Each was then divided into samples (4 ml.) which were mixed with varying amounts of saturated ammonium sulphate solution. The mixtures, and samples containing no ammonium sulphate, were kept for 24 hr. at room temperature and then centrifuged for 10 min. at 6000 rev./min.

Control solutions of the two viruses heated alone at a concentration of 0·042% coagulated completely and were sedimented by centrifugation without adding ammonium sulphate. The coagula from 4 ml. samples from the solutions of TMV and BSV contained 1·6 and 1·7 mg. of protein respectively. The coagulum obtained from the solution of TMV was free from nucleic acid, as it contained neither phosphorus nor carbohydrate. The coagulum from the solution of BSV, on the other hand, contained nucleic acid and had 1·3% phosphorus and 6% carbohydrate.

The presence of 0·25% albumin protected TMV from coagulation, and partially protected BSV. When solution *B* was left undisturbed after heating, a coagulum separated, but it formed much more slowly and was much less than in a control solution of the virus heated alone.

Fig. 2 shows that some protein was sedimented from solutions *A* and *B* by centrifugation for 10 min. at 6000 rev./min. in the absence of ammonium sulphate, and progressively more was sedimented with increasing amounts of the salt up to 33% saturation. Nothing was sedimented from the solution of albumin heated alone until the concentration of the salt exceeded 33% saturation.

All the virus was precipitated from solutions *A* and *B* when the ammonium sulphate concentration

reached 33 % saturation. With BSV this was shown by estimation of phosphorus, which constitutes 1·3 % of the virus and only about 0·01 % of the albumin. Thus solution *B* contained 0·022 mg. of virus phosphorus/4 ml. and only about 0·001 mg. of albumin phosphorus/4 ml. The total amount of protein in the precipitate was 4·5 mg. with a phosphorus content of 0·023 mg., so that the whole of the virus (1·7 mg.) must have been in the precipitate. Thus 2·8 mg. of albumin was combined into a complex with the virus.

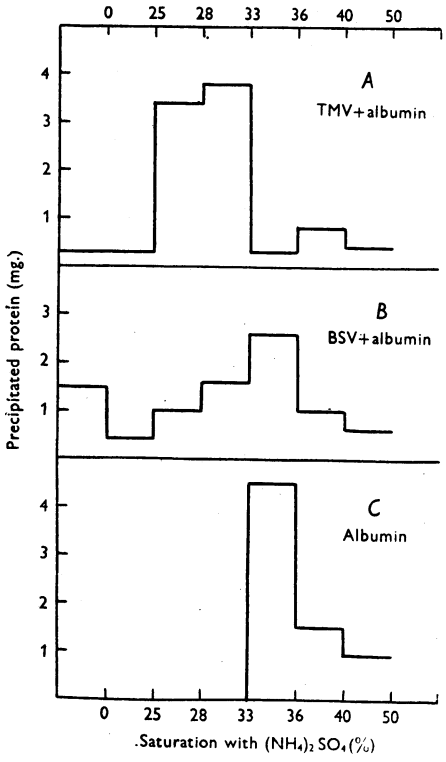


Fig. 2. Precipitating proteins by ammonium sulphate from heated solutions containing TMV, BSV and albumin. The ordinates show the increments in the amounts of precipitated protein corresponding to the increments in the concentration of ammonium sulphate shown by the abscissae. They were obtained by subtracting the amount of protein precipitated at a given concentration of the salt from the amount precipitated at the next higher concentration. *A*, 0·042 % TMV and 0·25 % albumin; *B*, 0·042 % BSV and 0·25 % albumin; *C*, 0·25 % albumin.

The content of TMV in the precipitate obtained from solution *A* by 33 % saturated ammonium sulphate was estimated colorimetrically by the May & Rose (1922) test for tryptophan, in which TMV gives four times the colour given by an equal weight of albumin. The possibility of an albumin fraction, with a chromogenic power different from the average, participating preferentially in the complex formation could be excluded. It can be seen from Fig. 2 that it is the fraction of heated albumin precipitable between 33 and 36 % saturation with ammonium sulphate which contributed mainly to the complex formation. This fraction was found to have the same chromogenic power as the remaining albumin.

The total amount of protein precipitated by 33 % saturated ammonium sulphate from heated solution *A* was 7·85 mg., which was equivalent in its chromogenic power to 3·2 mg. of TVM. Expressing the amount of virus in the precipitate as $x$, $x+(7{\cdot}85-x)/4=3{\cdot}2$, so that $x=1{\cdot}65$ mg., i.e. all the virus protein was in the precipitate, and so the complex contained 6·2 mg. of albumin.

From these results it follows that the albumin/virus ratios in the complexes were 3·9 and 1·65 with TMV and BSV, respectively. However, the fact that the complexes can be fractionated (Fig. 2) suggests that the ratio of the constituents in single aggregates of the complexes may have varied considerably, so that the ratios should be taken as averages for the two complexes.

The phosphorus content of TMV present in 4 ml. of the solution *A* was about 0·009 mg., whereas only a trace of phosphorus (about 0·001 mg.) could be detected in the precipitate obtained by 33 % saturation with ammonium sulphate. This can be attributed to the albumin present in the precipitate, as can the 0·06 mg. of carbohydrate found there (the carbohydrate content of the albumin is approx. 0·9 %). Thus the heat-denatured protein of the virus, which had formed a complex with heat-denatured albumin, did not contain any appreciable quantity of nucleic acid. The albumin, by forming a complex with the heat-denatured virus, can protect it from coagulation, but not from loss of nucleic acid. Similarly, it does not protect it from the loss of its ability to react with antibodies to the virus.

Bawden & Kleczkowski (1941, 1942*a*) showed that proteins such as BSV, which can be denatured by heat without losing their ability to react with their antibodies, if combined during heating into complexes with enough serum albumin, are not precipitated by their antibodies. They still combine with them, however, and can specifically inhibit the precipitation of unchanged antigens. By contrast, complexes formed during heating between serum albumin and TMV, which loses its serological activity during early stages of heat denaturation, do not inhibit precipitation of unchanged TMV. This has been confirmed with complexes formed during 10 min. heating at 80 or 83° in mixtures with albumin/TMV ratios varying from 5:1 to 15:1. No combination between the complexes and virus antibodies occurred to any extent demonstrable either by precipitation or by specific inhibition of precipitation of unchanged virus.

The denaturation rate of TMV does not seem to be influenced by the presence of the albumin. This conflicts with the earlier statement (Bawden & Kleczkowski, 1941) that addition of serum albumin protects TMV from the loss of serological activity and infectivity during heating. The solutions used in the previous work were made in 0·9 % sodium chloride and were unbuffered, and unnoticed differences in the pH between virus-albumin mixtures and solutions of the virus alone probably explain the apparent protection. In this work, with all the solutions containing M/15 phosphate buffer at pH 6·8, no differences in serological activities have been found between heated virus-albumin mixtures and solutions of the virus heated alone. At 83 or 80°, denaturation of TMV at pH 6·8 is so rapid that no comparisons can be made, but comparison is possible at 77°. Heating at 77° for varying lengths of time caused equal decreases in the precipitin titres, with an antiserum to TMV, in solutions of the virus alone and in those containing virus and albumin. This was so in spite of the fact that virus-albumin mixtures remained clear, whereas coagula appeared in the solutions of the virus alone.

*The effects of varying the ratio of the components in heated mixtures*

The complexes were isolated by precipitation with ammonium sulphate used at concentrations insufficient to precipitate control solutions of albumin

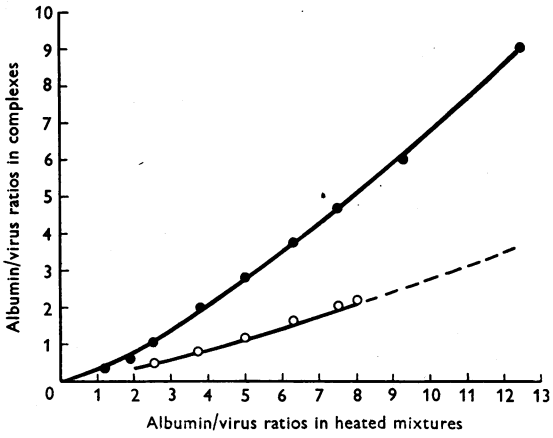


Fig. 3. Albumin/virus ratios in complexes formed in heated solutions containing the virus at constant concentration (0·04 %) and albumin in varying concentrations; ●, complexes between TMV and albumin; ○, complexes between BSV and albumin.

heated alone, and tests were made to ensure that all the virus was in the isolated complex. Fig. 3 shows the ratios of albumin to virus in the complexes formed in heated mixtures in which the virus concentration was kept constant at 0·04 % and that of the albumin varied. The method of isolating the complexes worked satisfactorily with the TMV-albumin mixtures over the whole range of the ratios shown. With BSV-albumin mixtures, however, it was satisfactory only until the albumin/virus ratio in the mixture reached 8. At ratios higher than 8 the complexes were not precipitated by ammonium sulphate until the salt concentration was raised to the level at which control albumin solutions also precipitated. Within the range tested, an increase in albumin/virus ratio in the heated mixture corresponded with a slightly more than proportional increase of the ratio in the complex formed. Thus, as the concentration of the albumin increased, so did the proportion of it combined with the virus into the complex (see Fig. 4).

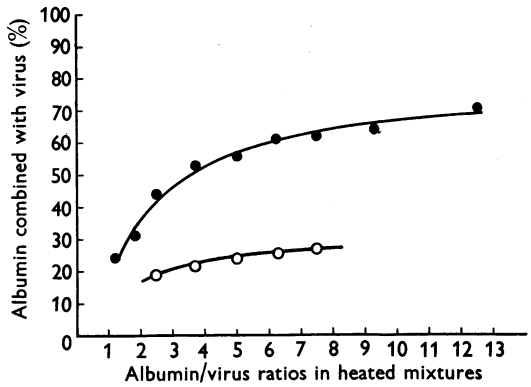


Fig. 4. Percentages of albumin combined into complexes with the virus in heated solutions containing the virus at constant concentration (0·04 %) and albumin in varying concentrations; ●, TMV; ○, BSV.

Figs. 3 and 4 also show that under similar conditions the amount of albumin combining with TMV was about 2·4 times that combining with BSV. The same amount of albumin combined with both viruses when the albumin/virus ratio in the heated mixture was about twice as great with BSV as with TMV. Similarly, when the two viruses were in equal concentrations, the minimum concentration of albumin necessary to protect BSV from heat coagulation was about twice as great as that necessary to protect TMV. For example, to protect 0·04 % BSV at least 0·3 % albumin had to be present, whereas with 0·04 % TMV 0·15 % albumin was sufficient. Under these conditions, the albumin/virus ratio in both complexes was about 1·8 and this ratio in the complex seems to be the minimum necessary to prevent heat coagulation of either virus.

When the albumin concentration in heated mixtures was kept constant and that of the virus varied, an increase in virus concentration was accompanied by a slightly less than proportional decrease in the albumin/virus ratio in the complex

(Table 1). From this, and from the results shown in Figs. 3 and 4, it can be deduced that, if the ratio of the components in heated mixtures is kept constant but the concentration is varied, the albumin/virus ratio in the complex should increase with the increase in the concentration. This was directly shown experimentally as described in the next section.

Table 1. *Effect of variation in virus concentration on the albumin/virus ratio in the complex*

| Composition of heated mixtures | | | | |
|---|---|---|---|---|
| TMV (%) | BSV (%) | Albumin (%) | Albumin/virus ratios in the heated mixtures | Albumin/virus ratios in the complexes |
| 0·04 | Nil | 0·5 | 12·5 | 9·0 |
| 0·08 | Nil | 0·5 | 6·25 | 4·6 |
| Nil | 0·04 | 0·5 | 12·5 | 3·8* |
| Nil | 0·08 | 0·5 | 6·25 | 2·2 |

* Value obtained by extrapolation in Fig. 3.

*The effect of simultaneous variation in the concentrations of both components in heated mixtures*

Fig. 5 shows the albumin/TMV ratios in the complexes formed in solutions containing both components at a constant ratio of 6:1 but at varying

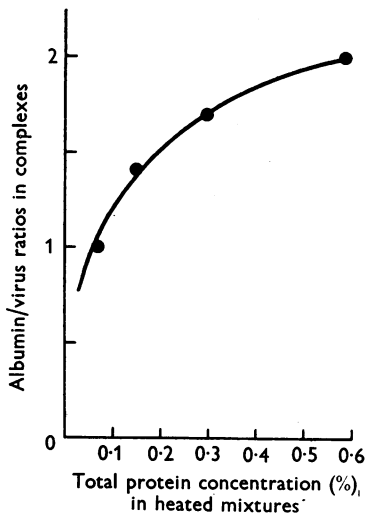


Fig. 5. The effect of total protein concentration in mixtures of albumin with TMV, at a constant ratio of the components (6:1), on the albumin/virus ratios in complexes formed during heating for 10 min. at 80°.

concentrations, and heated for 10 min. at 80°. It will be seen that as the total protein concentration increased, so did the albumin/virus ratio in the complex. Within the range tested, when the protein concentration increased geometrically, the ratio increased arithmetically.

Table 2 shows a comparison of the albumin/virus ratios in complexes formed by albumin with TMV and BSV, when the mixtures were heated at a constant ratio of the components but at two different concentrations. The ratio in the complexes formed with BSV depends on the total protein concentration as it does with TMV, although under the same conditions the ratios with BSV are smaller than those with TMV. Table 2 also shows that the ratios increase with increase in temperature.

Table 2. *Effect of total protein concentration on the ratio of the component proteins in the complexes*

| Composition of heated mixtures | | | Albumin/virus ratios in the complexes | |
|---|---|---|---|---|
| TMV (%) | BSV (%) | Albumin (%) | 10 min. at 80° | 10 min. at 83° |
| 0·04 | Nil | 0·25 | 1·7 | 3·7 |
| 0·08 | Nil | 0·5 | 2·0 | 4·6 |
| Nil | 0·04 | 0·25 | — | 1·6 |
| Nil | 0·08 | 0·5 | — | 2·0 |

Changes in the ratio of protein components in complexes, resulting from changes in protein concentration, can account for an apparently paradoxical phenomenon occasionally observed when protein mixtures are heated at different dilutions. Dilution, with ionic strength and pH kept constant, is one method of preventing coagulation of heated protein solutions, as the formation of large aggregates of protein particles is prevented. Occasionally, however, dilution before heating leads to coagulation during the heating. An example is shown in Table 3,

Table 3. *Effect of total protein concentration in mixtures of* BSV *with albumin on stability of heat-denatured virus*

| Concentration of constituents in heated mixtures | | | |
|---|---|---|---|
| BSV (%) | Albumin (%) | Appearance of the fluids after heating | Albumin/virus ratio in the complexes |
| 0·08 | 0·5 | Opalescent but transparent and stable | 2·0 |
| 0·04 | 0·25 | Small floccules settling slowly | 1·6 |
| 0·02 | 0·125 | Large floccules settling rapidly | 1·2 |

where an undiluted solution containing 0·08 % BSV and 0·5 % albumin did not coagulate after heating, but did if diluted twice or four times before heating.

When the albumin/virus ratio in the complex formed during heating was 2·0, the virus, at a concentration of 0·08 %, was protected from coagulation. When, because of diluting the mixture before heating, the ratio fell to 1·6 and 1·2, there was not enough albumin combined with the virus to prevent its coagulation, although the virus concentration fell to 0·04 and 0·02 %, respectively.

## DISCUSSION

This work was limited to complexes formed during heat denaturation between pairs of proteins, one of which when heated alone becomes insoluble while the other remains soluble. The ratio of the amount of the former to that of the latter in heated mixtures was limited to values less than 1. Of the factors likely to affect the composition and properties of complexes only variations in the concentration and in the ratio of the components in heated mixtures were studied.

Table 4. *Effect of concentration of heated albumin*-BSV *mixtures on serological properties of the complex*

(No coagulation appeared in any of the heated solutions. After cooling they were diluted (in saline) to bring the virus concentration to the values indicated. Precipitin test: 1 ml. of an antiserum to the virus at a dilution of 1/500 was added to 1 ml. of antigen solutions at various concentrations, and the mixtures placed in a water bath at 50°; + signs indicate the degree of precipitation; − signs indicate no precipitation where the test for inhibition was not made. Test for inhibition: 0·1 ml. of 0·05 % solution of unheated virus was added to the tubes where there was no precipitation after 3 hr. incubation; *i* indicates inhibition (no precipitation); 0 indicates no inhibition (precipitation).)

| Concentration of constituents in heated mixtures | | Albumin/virus ratios in the complexes | Precipitin test Virus concentration (g./100 l.) | | | | | |
|---|---|---|---|---|---|---|---|---|
| BSV (%) | Albumin (%) | | 10 | 5 | 2·5 | 1·25 | 0·625 | 0·312 |
| 0·08 | 1·0 | 4·4* | *i* | *i* | *i* | *i* | 0 | 0 |
| 0·04 | 0·5 | 3·7* | 0 | 0 | 0 | 0 | 0 | 0 |
| 0·02 | 0·25 | 3·0 | + + | + | 0 | 0 | 0 | 0 |
| 0·01 | 0·125 | 2·3 | + + + | + + + | + + | + | − | − |
| 0·005 | 0·0625 | 1·6* | . | + + + | + + | + | ± | − |
| Unheated virus (control) | | . | + + + | + + + | + + | + | ± | − |

* Values obtained by extrapolation from results of other experiments.

The decrease in the albumin/BSV ratio in the complex, resulting from reduced protein concentration, is also reflected in serological reactions of the complex with antibodies to the virus. The results of an experiment described in Table 4 show that when a solution containing 0·08 % BSV and 1 % albumin was heated, a complex was formed which contained over four times as much albumin as virus. The complex did not precipitate with virus antiserum and it inhibited precipitation of subsequently added unheated virus. When the concentration of the virus-albumin mixture was reduced by half before heating, the complex with albumin/virus ratio 3·7 gave no precipitate with the antiserum, and showed no inhibiting power. When the concentration of the virus-albumin mixture was still further reduced before heating, the ratio fell still further and, although there was still enough albumin in the complex to prevent coagulation, there was not enough to prevent precipitation with the antiserum to the virus. This agrees with previous work (Kleczkowski, 1941*b*) which has shown that formation of non-precipitating and inhibiting complexes between antibodies and other serum proteins in heated antisera can be prevented by increasing the dilution of the antisera in saline before heating.

The conclusions reached here can be expected to apply in general trends to similar protein systems, i.e. to pairs of proteins, one of which when treated alone becomes insoluble, whereas the other remains soluble, e.g. a serum globulin and a serum albumin. Numerical relationships true of one system are obviously inapplicable to another. For example, these differed in TMV-albumin and BSV-albumin systems, where complexes with different ratios of the components were formed under similar conditions. The general trends, however, were similar, for variations in the ratio and in the concentration of the components of the heated mixtures changed the ratios of the components in the complexes similarly in both systems.

A number of other factors, not studied in this work, are likely to influence the composition and properties of complexes formed between pairs of different proteins during heat denaturation. Among these are variations in the pH and in the character and concentration of salts present in heated solutions; it is known that complexes are not formed to any

appreciable extent in salt-free solutions, and that there is an interaction between the pH and the character and concentration of salts (Kleczkowski, 1943).

If the conclusions about the extent of aggregation of the albumin heated alone, deduced from the curves of precipitability by ammonium sulphate with the help of Smoluchowski's theory of aggregation (see Fig. 1), are at least approximately true, heat-denatured albumin molecules combine with heat-denatured virus particles much faster than among themselves. For example, in the heated 0·25 % albumin solution about 60, 30 and 20 % of the albumin existed as aggregates of at least two, three and four molecules respectively, and almost none as aggregates of seven or more molecules. By contrast, when mixtures containing 0·25 % of the albumin and 0·042 % of TMV or BSV were heated under the same conditions, about 60 and 25 % of the albumin formed a complex with TMV and BSV respectively. Particles of BSV are approximately spherical and equal in size, and there is no reason to suspect that they break into fragments during heat denaturation. Assuming that the weight of a particle of BSV, in terms of molecular weight, is $7 \times 10^6$, and that of the albumin molecule is $7 \times 10^4$, then the 25 % of the albumin would be combined into a complex with BSV with an average of 160 albumin molecules to one virus particle. Similar reasoning cannot be applied to TMV, the particles of which vary in size very considerably and undergo much greater changes during heat denaturation than do BSV particles. It is probable, however, that an average number of albumin molecules/virus particle in the albumin-TMV complex was even higher, perhaps 10 times, than that in the albumin-BSV complex. (For example, the ratio of 2000:1 would be obtained, if the average weight of TMV particles in the preparation used were taken as $5 \times 10^7$, and if it were further assumed that this was unaltered by heat denaturation.)

Two reasons can be given for aggregation of albumin molecules proceeding much faster with virus particles than among themselves. First, the proportion of collisions resulting in permanent combination may be higher in those between albumin molecules and virus particles than in those between albumin molecules themselves. Secondly, Wiegner (1911) and Galecki (1912) have shown that, in colloidal solutions containing particles of considerably different sizes, particles of greater sizes function as nuclei of aggregation for particles of smaller sizes. This is so because the probability of a collision between two particles, subjected to Brownian movement, is greater when they are of greatly different sizes than when they are of equal or not greatly different sizes (see Müller, 1928). The probability of a collision between two particles of the same size is independent of the size, and, when the sizes are not equal, it is increased by the factor

$$f = \frac{(R_1 + R_2)^2}{4R_1R_2},$$

where $R_1$ and $R_2$ are the radii (in any units) of the two particles, both assumed to be spherical. Thus, if heat-denatured albumin molecules and BSV particles are assumed to be approximately spherical and of the weights given above, the probability of a collision between an albumin molecule and a virus particle would be 1·7 times greater than between two albumin molecules or two virus particles. The probability of a collision of an albumin molecule with an 'average' particle of heat-denatured TMV is probably greater than with a particle of BSV.

## SUMMARY

1. The ratios of the constituent proteins in complexes formed by human-serum albumin with tobacco mosaic virus and with tomato bushy stunt virus during heat denaturation in solutions containing M/15 phosphate buffer pH 6·8, were studied.

2. When heated alone both viruses coagulate, whereas the albumin does not, although it aggregates increasingly with increasing concentration. When the albumin/virus ratio in the complex is 1·8 or higher, the complex forms a stable solution. All the virus and only part of the albumin participate in the formation of such a complex.

3. Heat-denatured tobacco mosaic virus, combined with albumin, contains no nucleic acid and has no serological activity, whereas the complex of bushy stunt virus retains both. When the albumin/bushy stunt virus ratio in the complex is over 3, the complex is not precipitable by virus antibodies although it combines with them.

4. At a constant virus concentration the albumin/virus ratios in the complex increase with increasing concentration of albumin; the ratios decrease with increasing virus concentration when the albumin concentration is kept constant; the ratios increase with increasing total protein concentration when the proportion of the constituents remains constant, and with increasing temperature when all other conditions are constant.

5. Under similar conditions about 2·4 times as much albumin combines into a complex with heat-denatured tobacco mosaic virus as with bushy stunt virus.

6. Heat-denatured albumin molecules aggregate more rapidly with particles of heat-denatured viruses than with one another.

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# Nitrogenous Excretion in Chelonian Reptiles

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It seems to be well established that, during the course of evolution, the migration of animals from aquatic to terrestrial environments has been at least partially dependent upon their ability to detoxicate the ammonia arising from the α-amino nitrogen of the food proteins (Baldwin, 1948). Among vertebrates, detoxication is usually accomplished by the formation of either urea or uric acid. Ureotelism occurs in the Amphibia and Mammalia, and uricotelism in the Sauropsida (snakes, lizards and birds).

The nitrogenous excretion of the Chelonian reptiles (tortoises and turtles) has not hitherto been systematically studied, and such data as are available are often contradictory. Magnus & Müller (1835) found evidence for the presence of urea in the urine of *Testudo nigrita* (*nigra*), while Schiff (1825) and Marchand (1845) similarly demonstrated the presence of urea in the urine of *T. denticulata* (*tabulata*). Both of these species are land dwellers. Several workers have studied *T. graeca*, another terrestrial form, Clementi (1929) stating that 90% of the excreted nitrogen is in the form of urea, an observation which is substantially supported by the results of Drilhon & Marcoux (1942). Münzel (1938), on the other hand, found that uric acid and urea were both produced in this species, the former predominating. He also analyzed the urine of the semi-aquatic species, *Emys orbicularis* (*europaea*) and found that the main execretory product was urea, uric acid accounting for only a small proportion of the nitrogen excreted. In both the species studied Münzel (1938) was able to demonstrate the synthesis of urea by liver slices, and, in *Testudo graeca*, of uric acid also. *Chrysemys picta*, which is also semi-aquatic, was studied by Wiley & Lewis (1927) who found that the main excretory product was urea, although ammonia and uric acid were also present in considerable quantities. Both Lewis (1918) and Khalil (1947) have worked on the sea turtle, *Chelonia mydas*, but obtained contradictory results. Lewis (1918) found that this species is essentially ureotelic, whereas Khalil (1947) states that ammonia is the main execretory product. Needham (1931), basing his argument on the work of Clementi (1929), Lewis (1918), and Wiley & Lewis (1927), concluded that Chelonia are essentially ureotelic. He believes that, because they are laid in a damp environment, the eggs can absorb water from the surrounding mud or sand and are therefore presumably permeable to urea also. In many species, however, the eggs are laid in very dry surroundings where a permeable egg would hardly be able to conserve or acquire enough water for development.

It therefore seemed worth while to investigate the nitrogenous excretory products of Chelonia from a variety of habitats in an attempt to clarify this somewhat obscure position. Species from a variety of aquatic, amphibious and terrestrial habitats were therefore studied to discover whether there might be some correlation between nitrogen metabolism and environmental conditions.

## EXPERIMENTAL

*Material.* Dr E. Hindle, F.R.S., was kind enough to put at our disposal the large collection of Chelonia belonging to the Zoological Society of London.

For the collection of urine the smaller specimens were kept and fed in rat metabolism cages of the usual type for 1 or 2 days. The wire floor separated the urine from the faeces, and fragments of food were removed by filtration through a loose plug of glass wool. The urine was collected in bottles containing a small amount of 0·2 N-$H_2SO_4$. The analysis