Holiday, E. R. (1945). *Biochem. J.* **39**, lxv.
Homer, A. (1915). *J. biol. Chem.* **22**, 345.
Jope, H. M. & Jope, E. M. Unpublished work.
Maillard, L. (1901). *Chem. Ztg*, **25**, 415.
Maillard, L. (1913). *Dictionnaire de physiologie*, cited by Fearon & Thompson. Paris.
Morton, R. A. & Stubbs, A. L. (1940). *J. chem. Soc.* p. 1347.
Nencki, M. & Sieber, N. (1882). *J. prakt. Chem.* **26**, 333.
Plimmer, R. H. A. (1920). *Practical Organic and Biochemistry*. London: Longmans, Green & Co.
Rosin (1891). *Arch. path. Anat.* **123**, 519.
Schunck, E. & Marchlewski, L. (1895*a*). *Ber. dtsch. chem. Ges.* **28**, 539.
Schunck, E. & Marchlewski, L. (1895*b*). *Ber. dtsch. chem. Ges.* **28**, 2525.
Schwartz, S., Marvin, J. F., Layne, J. A. & Watson, C. J. (1943). *Ann. intern. Med.* **19**, 206.
Sheppard, S. E. & Newsome, P. T. (1942). *J. Amer. chem. Soc.* **64**, 2937.
Watson, C. J. (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 514.
Watson, C. J. (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 591.
Watson, C. J. & Layne, J. A. (1943). *Ann. intern. Med.* **19**, 183.
Woodward, R. B. (1941). *J. Amer. chem. Soc.* **63**, 1123.

Combination between different Proteins and between Proteins and Yeast Nucleic Acid

By A. KLECZKOWSKI (Beit Memorial Research Fellow),
Rothamsted Experimental Station, Harpenden, Herts

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There are many substances that reversibly inhibit the infectivity of tobacco mosaic virus. The mechanism of inhibition is largely unknown, but some of the most effective inhibitors, trypsin and ribonuclease for example, combine with the virus (Kleczkowski, 1944; Loring, 1942). Other substances, such as clupein and nicotine, also combine with the virus (Bawden & Pirie, 1937*a*, *b*), though their effects on infectivity have not been tested. Combination between the virus and ribonuclease or clupein is apparent from the formation of visible precipitates when salt-free solutions of the components are mixed. The precipitates dissolve when neutral salt is added.

Precipitation of proteins by protamines, and of nucleic acids by proteins or protamines, is a well-known phenomenon that has been extensively studied (Ugglass, 1914; Steudel & Peiser, 1922; Hammarsten, 1924; Lissitzin & Alexandrowskaja, 1933). It is generally accepted to be the result of a salt-like combination occurring between oppositely charged substances. Similar phenomena have not been hitherto observed with pairs of proteins both having isoelectric points in the acid or in the alkaline region, and in the main they have also been confined to combinations between pairs of substances in solution, though it has been shown that suspensions of heat-coagulated egg albumin can combine with dissolved yeast nucleic acid (Przylecki & Grynberg, 1932), and also that solid yeast nucleic acid can combine with dissolved amylases (Butler, 1945). In the present paper it is shown that insoluble heat-coagulated proteins behave like proteins in solution and can combine with dissolved proteins, clupein and yeast nucleic acid. The conditions determining such combinations have been studied and attempts have been made to measure the effects of such combinations on the infectivity of tobacco mosaic virus.

MATERIAL AND METHODS

Purified tobacco mosaic virus, prepared by the method described by Bawden & Pirie (1943), was used.

Crude serum globulin was obtained from human serum by half-saturation with $(NH_4)_2SO_4$; the resulting precipitate was filtered off, washed in half-saturated $(NH_4)_2SO_4$, dissolved in H_2O and dialyzed against 0·9% NaCl.

Crude serum albumin was prepared from horse serum. After removing the globulin fraction by half-saturation with $(NH_4)_2SO_4$ and filtering, the albumin was precipitated by saturating the filtrate with $(NH_4)_2SO_4$. The precipitate was filtered off, dissolved in H_2O and dialyzed against H_2O.

Globin was prepared from rabbit haemoglobin. Blood corpuscles were sedimented from oxalated rabbit blood by centrifugation, washed several times with 0·9% NaCl and haemolyzed by adding distilled water. All sedimentable material was removed by centrifugation. Globin was prepared from the resulting solution by the HCl-acetone method of Anson & Mirsky (1930). The precipitate was washed thoroughly with acetone until washings were colourless, and dried.

Crystalline ribonuclease was prepared from beef pancreas by the method described by Kunitz (1940). The enzyme was twice crystallized from $(NH_4)_2SO_4$ solution, dialyzed against distilled water and dried.

Commercial preparations of clupein sulphate and of yeast nucleic acid were obtained from the British Drug Houses Ltd. About 40% of the nucleic acid was precipitated when 10 vol. of glacial acetic acid were added to 1 vol. of the neutral solution.

Suspensions of heat-coagulated proteins. 1% solutions of tobacco mosaic virus, globulin and globin in 1% NaCl were adjusted to pH 7·0 (which precipitated the globin), and heated for 5–10 min. in a water-bath at 100°. The coagulated proteins were centrifuged down and washed several times, first with water and then with the solutions in which they were finally suspended for the experiments. Fine suspensions were made by grinding first with small and then with greater quantities of the solutions. Suspensions of the denatured tobacco mosaic virus contained no phosphorus or carbohydrate, indicating the absence of nucleic acid.

Enzymic activity of ribonuclease. This was measured by estimating the amounts of yeast nucleic acid rendered unprecipitable by acetic acid. Samples to be tested were diluted in 0·2M-boric acid-borax buffer at pH 6·8 and mixed with equal volumes of 3% solutions of salt-free yeast nucleic acid at pH 6·8. The mixtures were incubated in a water-bath at 50° for 30 min., and 10 ml. of glacial acetic acid were then added to 1 ml. of the mixture. The fluids were centrifuged, the precipitates washed in acetic acid and their phosphorus contents measured. Several control experiments with known but varying amounts of the enzyme (usually about 0·01 mg./ml. and less) were made simultaneously under the same conditions, and it was found that the amounts of phosphorus rendered unprecipitable by acetic acid increased with increasing concentrations of ribonuclease in a non-linear manner. Estimates of the ribonuclease in test fluids were made by graphic interpolation using the data obtained in the control experiments.

Other estimations. Nitrogen was determined by the micro-Kjeldahl method, phosphorus and carbohydrate colorimetrically, using for the former a method based on the reduction of phosphomolybdic acid by stannous chloride in the presence of H_2SO_4 and for the latter an orcinol method (see Snell & Snell, 1936, 1937). To suit the requirements of this work a modified method for estimations of phosphates in water was used. Materials under test were first incinerated with H_2SO_4 and cleared by adding a few drops of perhydrol; the fluids were then diluted in water and mixed with a 12·8% (w/v) solution of ammonium molybdate so that the proportions of H_2SO_4 and ammonium molybdate corresponded to those obtaining in the 'Molybdate reagent'.

Infectivity tests with tobacco mosaic virus were made by the local lesion method using *Nicotiana glutinosa* as host plant. The half-leaves inoculated with each preparation were randomized in a Latin square design and at least eight leaves were used for each treatment. Inoculation was effected by rubbing the leaf with the forefinger and afterwards washing with water.

RESULTS

Isoelectric points of the proteins

To test whether the electric charge determines combination, it was necessary to know, at least approximately, the isoelectric points of the proteins. The isoelectric points of tobacco mosaic virus and unheated globin, and of suspensions of heat-coagulated globin, serum globulin and the protein component of tobacco mosaic virus, were determined by finding the pH at which the materials gave maximal flocculation. The suspensions are uniform and settle very slowly, except in pH ranges around their isoelectric points when they settle quickly with formation of large floccules.

Solutions or suspensions (0·1%) were made in distilled water and placed in a number of narrow tubes. The pH was adjusted to different values by adding HCl or NaOH and measured with a glass electrode. (Buffer solutions were not used, as it is known that the presence of salts may influence the results (Michaelis & Rona, 1919; Michaelis & Szent-Györgyi, 1920), particularly when they contain polyvalent ions.) The pH of the fluid in the tube that first showed obvious floccules was taken as the isoelectric point. The results are shown in Table 1. Differences in the pH values between successive tubes were rather large, so the values can only be considered as approximations.

Tobacco mosaic virus was tested in the presence of 0·5% NaCl, as in salt-free solutions its maximal flocculation is difficult to detect. According to Bawden & Pirie (1937*a*) the virus precipitates optimally in the presence of salt at pH 3·4, and in salt-free solutions, or with a salt concentration of less than M/50, at about pH 4·2. The pH value of about 3·2 shown in Table 1 agrees with these results and with the isoelectric point of the virus found electrophoretically in the presence of $(NH_4)_2SO_4$ by Loring & Stanley (1937).

Table 1. pH *values at optimal flocculation of the proteins tested*

Protein	pH at optimal flocculation
Solution of intact tobacco mosaic virus*	3·2
Suspension of heat coagulated protein of tobacco mosaic virus	5·0
Suspension of heat coagulated globulin	6·0
Solution of unheated globin	7·5
Suspension of heat coagulated globin	7·5

* The preparation contained 0·5% NaCl.

The isoelectric point of unheated and of heat-coagulated rabbit globin was found to be pH 7·5. Isoelectric points of globins of other species also have been found to be in the alkaline region, that of bovine globin, for example, pH 8·1 (Osato, 1922).

The isoelectric point of the heat-coagulated protein component of tobacco mosaic virus was found to be pH 5·0, and that of heat-coagulated human serum globulin pH 6·0. Proteins with isoelectric points below pH 7·0 usually shift their isoelectric points by not more than 0·5 of the pH unit towards the alkaline side as a result of denaturation (Neurath, Greenstein, Putnam & Erickson, 1944). The much greater shift found with tobacco mosaic virus is almost certainly a result of removing the nucleic acid from the protein component.

Whole-serum globulin in its native state contains several fractions with different isoelectric points. Stenhagen (1938) found three, designated α, β and γ, with isoelectric points at about pH 4·8, 5·2 and 6·4 respectively. The fact that the suspension of heat-coagulated whole-serum globulin seemed to have its isoelectric point at about pH 6·0 can be explained as a result of the formation of complexes when different proteins undergo heat denaturation together in the presence of salt (Kleczkowski, 1941, 1943, 1945). Different fractions of native globulin, with different isoelectric points, would combine during heat denaturation to form a complex, and all particles of the resulting suspension would have either the same isoelectric point, or different isoelectric points but distributed over a much narrower pH range than those of the separate fractions in native globulin.

Tiselius (1937), working with whole serum and also with an electrophoretically isolated albumin fraction, found horse-serum albumin to be isoelectric at pH 4·64, and Kekwick (1938) found the isoelectric point of crystalline fractions to be pH 4·8. The isoelectric point of ribonuclease was given by Rothen (1940) as pH 7·8, and that of clupein by Miyake (1927) as pH 12·1.

Yeast nucleic acid contains free amino groups in guanine, adenine and cytosine, so that it may be amphoteric. It was assumed, however, that, in the conditions in which it was used in this work, i.e. at pH values higher than those at which it precipitated, it was always negatively charged.

Combinations between materials mixed as solutions

In Table 2 the behaviour of tobacco mosaic virus on mixing with solutions of proteins, clupein or yeast nucleic acid is compared with that of other proteins. Equal volumes of 0·2% solutions of the tested materials at the required pH (adjusted with HCl or NaOH and measured with a glass electrode) were mixed. Except for serum globulin with which tests were made in M/100 NaCl, all fluids were salt-free. At pH 4·7 globulin solutions became turbid, presumably because the fraction isoelectric near this pH (Stenhagen, 1938) was precipitated, and about 20% of the protein could be centrifuged off. The clear supernatant fluid was used in the tests. Tobacco mosaic virus, serum globulin and globin are insoluble at pH values around their isoelectric points, and consequently their precipitability by other substances could not be tested at these pH values.

It will be seen that precipitation occurred in most mixtures when the pH was such that the constituents carried opposite electric charges. The behaviour of tobacco mosaic virus did not differ from that of other proteins. The isoelectric points of globin and clupein are both in the alkaline region,

Table 2. *The effect of* pH *on mutual precipitation of proteins and nucleic acid*

(pH values of isoelectric points of the proteins, or a pH range within which isoelectric points of component fractions are distributed, are given in brackets.)

Mixture	pH of the mixture	Precipitation
TMV (4·2) + RNase (7·8)	2·5	–
	4·7	+ + + +
	6·0	+ + + +
	6·5	+ + +
	7·0	+ +
	7·5	–
	8·0	–
TMV (4·2) + clupein (12·1)	2·5	–
	4·7	+ + +
	6·5	+ + + +
	9·0	+ + + +
TMV (4·2) + globin (7·5)	2·5	–
	4·7	+ + +
	6·5	+ + + +
	9·0	–
TMV (4·2) + globulin (4·8–6·4)	2·5	–
	4·7	+ +
	6·5	–
	9·0	–
Globulin (4·8–6·4) + clupein (12·1)	4·0	–
	4·7	–
	6·5	+ +
	9·0	+ + +
Globulin (4·8–6·4) + globin (7·5)	4·0	–
	4·7	–
	6·5	+ +
	9·0	–
Albumin (4·8) + RNase (7·8)	4·0	–
	6·0	–
	9·0	–
Clupein (12·1) + RNase (7·8)	6·0	–
	9·0	–
Globin (7·5) + RNase (7·8)	6·0	–
	9·0	–
Globulin (4·8–6·4) + clupein (12·1)	4·5	–
	6·5	+
	8·0	–
Albumin (4·8) + clupein (12·1)	4·5	–
	4·9	–
	5·1	±
	5·5	+
	6·0	+ +
	6·4	+ + +
	9·0	+ + +
Albumin (4·8) + globin (7·5)	4·5	–
	4·9	–
	5·1	±
	5·5	+
	6·0	+ +
	6·5	+ +
	9·0	–
Globin (7·5) + clupein (12·1)	6·5	–
	9·0	+ + +
Albumin (4·8) + NA	4·4	+ +
	4·6	+
	4·8	–
	5·0	–
Globulin (4·8–6·4) + NA	4·0	+ + +
	4·7	+
	6·5	–
Globin (7·5) + NA	6·0	+ + +
	9·0	–
Clupein (12·1) + NA	6·0	+ + + +
	9·0	+ + + +
TMV (4·2) + NA	2·5	+
	4·7	–
RNase (7·8) + NA	6·0	+
	8·0	–

TMV, tobacco mosaic virus; RNase, ribonuclease; NA, yeast nucleic acid.
Plus signs indicate appearance and degree of turbidity followed by precipitation.

yet these also co-precipitated when the pH of the mixture was intermediate between the isoelectric points. Similarly, tobacco mosaic virus could be precipitated by serum globulin, although the isoelectric points of both are in the acid region. When examined microscopically this precipitate was found to consist of para-crystalline threads similar to those produced by combination between the virus and ribonuclease or clupein, whereas the precipitate formed with globin seemed to be amorphous. When the NaCl concentration was raised to M/50, most of the precipitate with globulin dissolved, and at still higher salt concentrations it dissolved completely.

A minute precipitate is also produced when the virus and yeast nucleic acid are mixed at pH 2·5; i.e. on the acid side of the isoelectric point of the virus. This precipitate settles slowly when left undisturbed and leaves a clear supernatant fluid. It is amorphous and dissolves in 1% NaCl solutions. The pH range at which this precipitate can be obtained is rather limited, as at pH 2·5 yeast nucleic acid begins to be slightly opalescent, and the opalescence increases considerably when the pH is lowered to 2·0.

All the precipitates shown in Table 2, except that formed between globin and clupein, are dissolved by adding NaCl to a concentration of 2%.

Table 2 shows that mixtures of some pairs of substances did not precipitate at any pH value, although their isoelectric points are as far removed from each other as those of other materials which did precipitate. For example, there was no precipitation in albumin-ribonuclease mixtures, or in clupein-ribonuclease mixtures.

Combinations of tobacco mosaic virus with ribonuclease, globin and clupein at pH 6·0 and the effect of NaCl on these combinations, were studied quantitatively. At this pH, particles of the virus are negatively charged, whereas the other three materials are positively charged. In salt-free mixtures precipitates are formed which can be dissolved by adding sufficient NaCl. Tests were made to see whether the addition of NaCl sufficient to dissolve the precipitates splits the compounds, or whether it merely makes them soluble.

Salt-free solutions of the virus were mixed with those of the other materials at pH 6·0. One set of mixtures was kept salt-free, whereas NaCl was added to another. Precipitates were formed immediately the solutions were mixed, and these dissolved immediately NaCl was added. In the set without NaCl, precipitates were removed by centrifugation for 5 min. at 8000 r.p.m. In the set to which NaCl had been added the mixtures were centrifuged for 1 hr. at 40,000 r.p.m., which is sufficient to sediment the virus, but not any of the other constituents of the mixtures, when free. The amounts of the constituents were then estimated in the supernatant fluids and/or in the sediments. The details and results of the experiment are given in Table 3. It will be seen that in the absence of salt most of the globin and ribonuclease, and about one-third of the clupein, were combined with the virus. The addition of enough NaCl to dissolve all the precipitates, split the combinations with globin and with ribonuclease to a considerable extent, and that with clupein to a smaller extent. The inference is that the virus-clupein compound is less susceptible to NaCl than the other two. It can be noticed, however, that none of the combinations was split completely, although the precipitates were all dissolved.

Table 3. *The effect of* NaCl *on combination of tobacco mosaic virus with clupein, globin and ribonuclease*

Materials mixed in 7·4 ml.	The volumes made up to 8·0 ml. by adding	Further treatment	N and P contents (in mg.) of Supernatant fluid N	Supernatant fluid P	Sediment N	Sediment P	TMV in the sediment* (%)	Other material in the sediment* (%)
16·0 mg. TMV + 4 mg. clupein	Water	Centrifuged	0·45	0	2·66	0·077	100	30
16·0 mg. TMV + 4 mg. globin		for 5 min. at	0·055	0	2·97	0·077	100	91
16·0 mg. TMV + 4 mg. ribonuclease		8000 r.p.m.	0·47	0·009	2·56	0·068	88	70†
16·0 mg. TMV + 4 mg. clupein	20% (w/v)	Centrifuged	0·52	0	2·58	0·077	100	19
16·0 mg. TMV + 4 mg. globin	NaCl	for 1 hr. at	0·50	0	2·52	0·077	100	11
16·0 mg. TMV + 4 mg. ribonuclease		40,000 r.p.m.	0·54	0	2·48	0·077	100	5†
16·0 mg. TMV	Water or	Centrifuged	0	0	2·46	0·077	100	—
4·0 mg. clupein	20% (w/v)	for 1 hr. at	0·64	—	0	—	—	0
4·0 mg. globin	NaCl	40,000 r.p.m.	0·56	—	0	—	—	0
4·0 mg. ribonuclease			0·57	—	0	—	—	0

TMV, tobacco mosaic virus.

* Computed from the distributions of N and P between the sediments and the supernatant fluids.

† By estimation of its enzymic activity 70% of ribonuclease was found in the sediment and 30% in the supernatant fluid. Similarly 5% of ribonuclease was found in the sediment and 95% in the supernatant fluid.

A similar experiment was made to test combination of the virus with the three materials at pH 9·0. Salt-free solutions of the components were adjusted to pH 9·0 and mixed in the same proportions as in the experiment shown in Table 3. No precipitates formed in the mixtures of the virus with ribonuclease or globin. The virus was sedimented from these mixtures by centrifugation for 1 hr. at 40,000 r.p.m. A precipitate formed in the mixture of the virus with clupein, and this was removed by centrifugation for 5 min. at 8000 r.p.m. The amounts of the components in the sediments and in the supernatant fluids were estimated as in the experiment shown in Table 3. The virus-clupein mixture gave the same results at pH 9·0 as it gave at pH 6·0. About 30% of clupein was in the precipitate combined with the virus. The results with ribonuclease and with globin, however, were different. Only about 20% of ribonuclease, and almost no globin, were found in the sediments in combination with the virus.

The results suggested that a test of the effect of NaCl and of the pH on inhibition of infectivity of the virus by ribonuclease, globin and clupein would be of interest. To test the effect of NaCl, salt-free solutions of the virus were mixed with those of the other materials. The solutions were at pH 6·0. One set of the solutions was left salt-free and NaCl up to a concentration of 0·3M was added to another set. All the mixtures were then inoculated into *N. glutinosa*. The details and the results of the experiments are shown in Table 4. It will be seen that all three materials reduced the infectivity of the virus. Ribonuclease is a much more powerful inhibitor than globin or clupein, while globin is rather more effective than clupein. To get comparable reduction of infectivity with these inhibitors ribonuclease must be used at 1/100th of the concentration of the other two substances.

Table 4 shows that the addition of NaCl to the virus reduced the numbers of lesions given by both the control virus solutions and mixtures of the virus with ribonuclease and with clupein. Thus direct interpretation of the effect of the salt on the inhibition of infectivity of the virus by these two materials cannot be made. With globin, however, the salt obviously reduced the inhibition considerably, for when added to the virus-globin it led to the formation of more lesions than were given by the salt-free mixture. That salt also reduced the inhibiting effects of ribonuclease and clupein is strongly suggested by the fact that, if the numbers of lesions given by the mixtures of the virus with the two materials are treated as percentages of those given by the respective controls, the addition of NaCl increased the percentages.

Table 4. *The effect of* NaCl *on inhibition of infectivity of tobacco mosaic virus by ribonuclease, globin and clupein*

(All solutions at pH 6·0.)

Contents of the mixtures/ml.	Added 1/10th vol. of	Average numbers of lesions per leaf	Numbers of lesions as % of controls
0·1 mg. TMV + 0·01 mg. ribonuclease	Water	27	10
+ 0·1 mg. ribonuclease		3	1
+ 1·0 mg. globin		14	5
+ 1·0 mg. clupein		62	23
Water control		266	100
+ 0·01 mg. ribonuclease	20% (w/v) NaCl	17	16
+ 0·1 mg. ribonuclease		2	2
+ 1·0 mg. globin		20	20
+ 1·0 mg. clupein		44	41
Water control		107	100
0·01 mg. TMV + 0·01 mg. ribonuclease	Water	1·75	4
+ 0·1 mg. ribonuclease		0	0
+ 1·0 mg. globin		1	2·5
+ 1·0 mg. clupein		8	19
Water control		42	100
+ 0·01 mg. ribonuclease	20% (w/v) NaCl	2·5	12·5
+ 0·1 mg. ribonuclease		0·5	2·5
+ 1·0 mg. globin		6	30
+ 1·0 mg. clupein		7	35
Water control		20	100

The effects of all three materials on infectivity of the virus can be reversed by dilution. The more the mixtures are diluted in water, the nearer the numbers of lesions obtained approximate to those formed by equally diluted control virus solutions.

To test the effect of pH on inhibition, salt-free mixtures of the virus with globin, clupein and ribonuclease were made at pH 6·0 and pH 9·0, and the mixtures were inoculated into *N. glutinosa*. The details and the results are shown in Table 5. It will be seen that clupein inhibited infectivity of the

Table 5. *The effect of* pH *on inhibition of infectivity of tobacco mosaic virus by ribonuclease, globin and clupein*

Contents of the mixtures/ml.	pH	Average numbers of lesions per leaf	Numbers of lesions as % of controls
0·1 mg. TMV + 0·01 mg. ribonuclease	6·0	15	12
+ 1·0 mg. globin		6	5
+ 1·0 mg. clupein		25	20
Water control		127	100
+ 0·01 mg. ribonuclease	9·0	23	18
+ 1·0 mg. globin		33	26
+ 1·0 mg. clupein		27	21
Water control		126	100
0·01 mg. TMV + 0·01 mg. ribonuclease	6·0	2	6
+ 1·0 mg. globin		0·5	1·5
+ 1·0 mg. clupein		5	16
Water control		32	100
+ 0·01 mg. ribonuclease	9·0	5	15
+ 1·0 mg. globin		5	15
+ 1·0 mg. clupein		4	12
Water control		33	100

virus at pH 9·0 as strongly as at pH 6·0, whereas ribonuclease and globin inhibited at pH 6·0 much more strongly than at pH 9·0.

Combination of dissolved proteins and yeast nucleic acid with suspensions of heat-coagulated proteins

Suspensions of heat-coagulated protein can be flocculated by yeast nucleic acid or clupein in the same manner as dissolved protein can be precipitated by the two materials. Table 6 shows the behaviour of salt-free mixtures containing 0·1% protein suspensions and 0·05% solutions of clupein or nucleic acid. The constituent fluids were adjusted to desired pH values with HCl or NaOH before they were mixed. All tests were made at the pH values remote from the isoelectric points of the protein suspensions, so that flocculation in the controls was slow. Mixing with clupein or nucleic acid caused rapid formation of large floccules which quickly settled. This occurred only at pH values at which the protein suspensions and the solutions of nucleic acid or clupein were oppositely charged. Suspensions of globin and of denatured protein from tobacco mosaic virus also flocculated one another at pH 6·0, i.e. when their particles were oppositely charged.

The presence of 2% NaCl did not prevent flocculation in the mixtures of protein suspensions with nucleic acid or clupein, but floccules were formed much more slowly than in the absence of salt.

Suspensions of heat-coagulated rabbit globin, human-serum globulin and the protein component of tobacco mosaic virus were tested quantitatively at different pH values for their ability to combine with solutions of yeast nucleic acid, ribonuclease, clupein and horse-serum albumin, respectively. The adsorption of the same materials by charcoal was also tested. The suspensions of heat-coagulated proteins and charcoal were made in M/50 solutions of buffers at different pH values and mixed with solutions of materials tested for combination in the same buffer solutions. In the tests with clupein at pH values 6·0 and higher, buffers could not be used,

Table 6. *Flocculation of suspensions of heat-coagulated proteins by oppositely charged materials*

(The figures in brackets indicate isoelectric points of the materials.)

Mixture	pH	Flocculation	Mixture	pH	Flocculation
Globulin (susp.) (6·0) + clupein	3·5	−	Globin (susp.) (7·5) + clupein	5·0	−
(sol.) (12·1)	7·5	+	(sol.) (12·1)	9·5	+
Globulin (susp.) (6·0) + NA (sol.)	3·5	+	Globin (susp.) (7·5) + NA (sol.)	5·0	+
	7·5	−		9·5	−
TMV prot. (susp.) (5·0) + clupein	3·5	−	TMV prot. (susp.) (5·0) + globin	3·5	−
(sol.) (12·1)	7·5	+	(susp.) (7·5)	6·0	+
TMV prot. (susp.) (5·0) + NA (sol.)	3·5	+		9·5	−
	7·5	−			

TMV prot., heat-coagulated protein component of tobacco mosaic virus; NA, yeast nucleic acid; (susp.), suspension of a heat-coagulated protein; (sol.), solution.

because clupein sulphate becomes opalescent and precipitates. The following buffer solutions were used: pH 2·0–4·9, Sorensen's citrate-HCl; pH 5·0–6·6, Sorensen's citrate-NaOH; pH 6·8–9·2, boric acid-borax; pH 9·2–11·0, borax-Na_2CO_3. When testing clupein at pH 6·0 or higher, the fluids contained M/50 NaCl and the pH values were adjusted by adding NaOH and measured with a glass electrode.

The mixtures usually contained about 0·5% of suspended materials and 0·05–0·1% solutions of the materials to be tested for combination with them. Albumin was used at 0·05%, ribonuclease at 0·07% and clupein and nucleic acid at 0·1%. After mixing, the fluids were centrifuged for 5 min. at 8000 r.p.m., and the amount of the material under test was measured in the supernatant fluids and/or in the sediments.

The tested materials combined with the suspensions of heat-coagulated proteins rapidly, so that there was no appreciable difference in the results whether the mixtures were centrifuged immediately after having been mixed, or whether they were centrifuged after standing for 1 hr. at room temperature. On the other hand, adsorption by charcoal proceeded more slowly; it was usually nearly complete after 20 min. at room temperature with occasional stirring of the fluids; in all the tests for adsorption by charcoal, therefore, the mixtures were centrifuged after a standard period of 20 min.

The amounts of horse-serum albumin or of clupein that combined with the suspended materials were determined by estimating nitrogen in the supernatant fluids, and those of the nucleic acid by estimating phosphorus. (In control experiments, where the suspensions were centrifuged alone, no nitrogen or phosphorus was present in the supernatant fluids.) Ribonuclease was estimated by measuring its enzymic activity. This was done both with the supernatant fluids and the sediments. The sediments were suspended in volumes of water equal to those of the fluids from which they were centrifuged. Before testing, both the supernatant fluids and suspended sediments were diluted at least 1/50 in 0·2M-solutions of boric acid-borax buffer at pH 6·8 for tests of enzymic activity. At such dilutions there was no detectable interference with enzymic activity of ribonuclease by the other constituents of the sediments.

The results of the experiments are shown graphically by continuous lines in Fig. 1. It can be seen that suspensions of all three heat-coagulated proteins combined with the nucleic acid, but only at pH values on the acid side of their isoelectric points, i.e. when their particles carried an electric charge opposite to that carried by the nucleic acid.

All three protein suspensions combined with clupein, but only at pH values on the alkaline side of their isoelectric points, i.e. again only when they were charged oppositely from clupein.

Similarly, heat-coagulated suspensions of globulin and of the protein from tobacco mosaic virus combined with ribonuclease only on the alkaline side of their isoelectric points. Maximum combination occurred at a pH value between the isoelectric point of the heat-coagulated protein and that of ribonuclease. There was still considerable combination at the isoelectric point of ribonuclease (pH 7·8), i.e. when the protein suspensions were charged negatively and ribonuclease carried no net charge. There was also some combination above the isoelectric point of ribonuclease, although the amount combined decreased rapidly as the pH increased. This can be explained as a result of attraction between negatively charged protein suspensions and ribonuclease which carries no net charge, as in the vicinity of the isoelectric point of ribonuclease some molecules carry no net charge and the proportion of such molecules decreases rapidly as the pH is shifted away from the isoelectric point.

Suspensions of heat-coagulated globin did not combine with ribonuclease to any detectable extent at any pH value. This can be explained by the close proximity of their isoelectric points, so that there is no pH value at which electrostatic forces exist to cause combination.

No combination between serum albumin and any of the three heat-coagulated proteins could be detected at any pH value. Absence of sufficiently strong electrostatic forces of attraction could explain lack of combination of albumin with the suspensions of globulin and of protein of the virus, for their isoelectric points are near that of the albumin. On the other hand, no such explanation could be offered to account for lack of combination between albumin and the suspensions of heat-coagulated globin, for their isoelectric points differ considerably.

None of the three heat-coagulated proteins, when used as isoelectric suspensions, combined to any appreciable extent with any of the tested materials. An isoelectric suspension of native tobacco mosaic virus (in M/50 buffer solution at pH 3·3) was tested similarly for combination with solutions of nucleic acid, clupein, globin, and ribonuclease, and no combination was detected.

Combinations of the suspensions of heat-coagulated proteins with solutions of other proteins or of nucleic acid can be split to a considerable extent, though not entirely, by 0·3M-NaCl. This can be seen from the results of an experiment given in Table 7.

The effect of pH and of the presence of 0·3M-NaCl on adsorption of yeast nucleic acid, ribonuclease, clupein and serum albumin by charcoal are shown in Fig. 1. Adsorption in the presence of 0·3M-NaCl

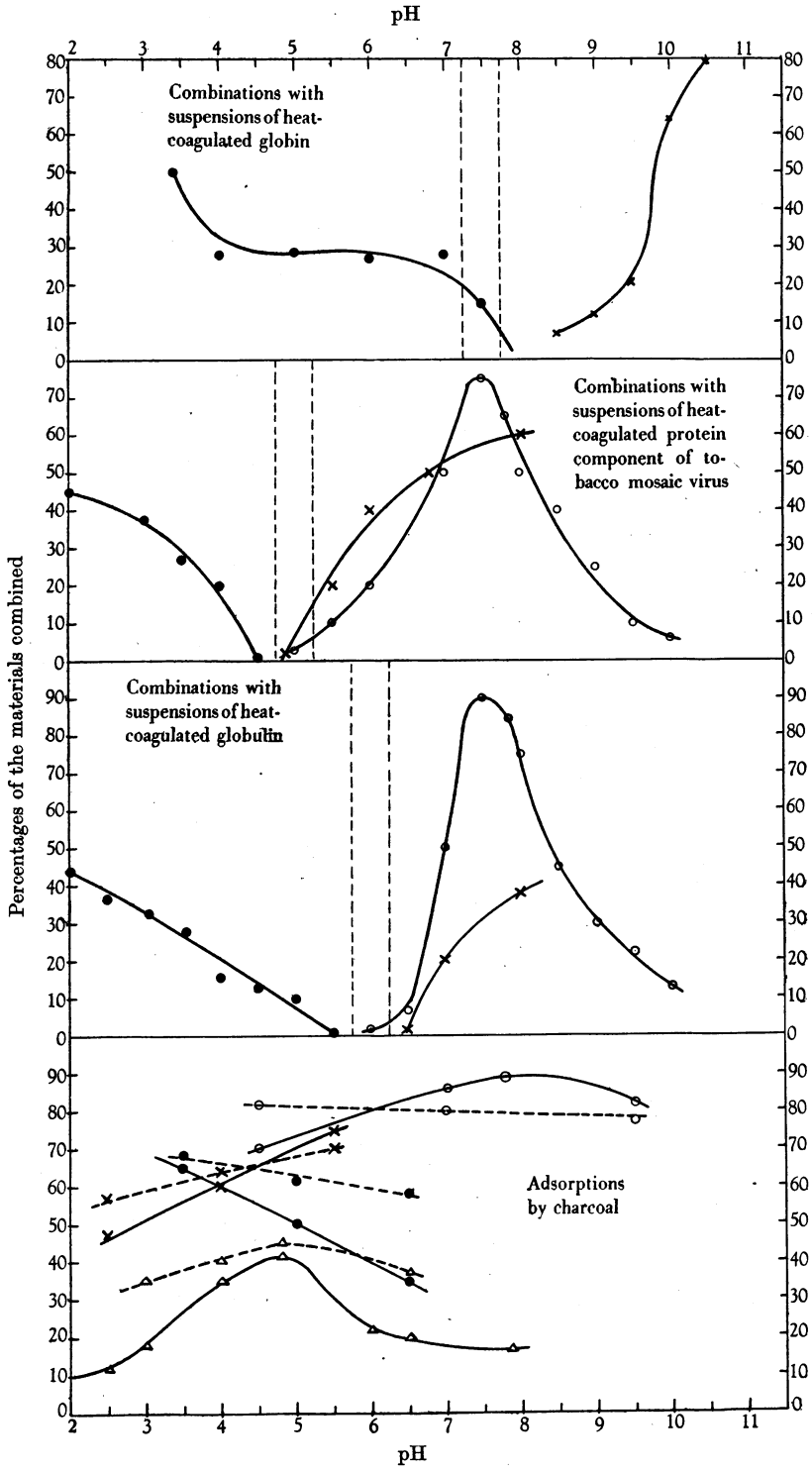

Fig. 1. The effect of pH on the combination of yeast nucleic acid, ribonuclease, clupein and horse-serum albumin in solution with suspensions of heat-coagulated proteins and of charcoal. • Yeast nucleic acid. o Ribonuclease. × Clupein. △ Horse-serum albumin. —— No NaCl added. - - - - 0·3M-NaCl added. Perpendicular broken lines show probable limits of the isoelectric points of the heat-coagulated protein suspensions.

Table 7. *The effect of* NaCl *on combination of heat-coagulated protein suspensions with ribonuclease, clupein and yeast nucleic acid*

(The suspensions were sedimented from the mixtures by centrifugation for 5 min. at 8000 r.p.m., and the amounts of the other components, which remained in the supernatant fluids were estimated.)

Contents of the mixtures/ml.				Percentage of the 2nd component combined with the suspension of the 1st component
1st component: Suspension of heat denatured protein	2nd component: Solution of	pH of the mixtures	Added 1/10th vol. of	
5 mg. protein of TMV	0·5 mg. RNase	7·0	Water	83
		7·0	20% NaCl	3
5 mg. globulin	0·5 mg. RNase	7·0	Water	96
		7·0	20% NaCl	12
5 mg. protein of TMV	1·0 mg. clupein	8·0	Water	61
		8·0	20% NaCl	43
5 mg. globulin	1·0 mg. clupein	8·0	Water	36
		8·0	20% NaCl	21
5 mg. globin	1·0 mg. clupein	10·0	Water	64
		10·0	20% NaCl	50
5 mg. protein of TMV	0·5 mg. NA	3·4	Water	38
		3·4	20% NaCl	3
5 mg. globulin	0·5 mg. NA	3·4	Water	38
		3·4	20% NaCl	3
5 mg. globin	0·5 mg. NA	3·4	Water	50
		3·4	20% NaCl	3

TMV, tobacco mosaic virus; RNase, ribonuclease; NA, yeast nucleic acid.

is shown by broken lines and in the presence of M/50 buffer solutions alone by continuous lines. All the materials were adsorbed at all pH values tested, although the amount of adsorption depended on the pH. Adsorption of negatively charged materials decreased with the increasing pH, whereas the reverse was true for positively charged materials. Serum albumin and ribonuclease were absorbed most at, or near, their isoelectric points. These results agree with those of Abderhalden & Fodor (1920), who studied the effect of pH on adsorption of a yeast protein by charcoal. It seems, therefore, that the sign of the electric charge of a given material does not influence its adsorption by charcoal. The presence of electric charge, however, irrespective of its sign, interferes with the adsorption, which decreases with increasing charge.

The presence of 0·3 M-NaCl lessens the dependence of adsorption on the pH. In the pH ranges where adsorption was well below the maximum, the presence of NaCl increased adsorption. On the other hand, in the pH ranges of maximum adsorption, the presence of NaCl either increased adsorption only slightly, or sometimes caused a slight decrease. The effect of pH and of salts on adsorption of proteins by charcoal is similar to that found by Hitchcock (1925) and Dow (1935–6) for adsorption of gelatin and egg albumin by collodion membranes.

DISCUSSION

The results described in this paper show that combination between proteins, or between proteins and nucleic acid, is a fairly general phenomenon and can be expected whenever two are mixed at pH values at which they are oppositely charged. Widely differing isoelectric points are not essential and combination was demonstrated between proteins both of which have isoelectric points on the same side of neutrality.

These combinations are often evident from precipitation of the compounds formed. The formation of precipitates is probably a result of mutual discharge, as are flocculations in mixtures of oil-water emulsions with oppositely charged protein solutions (Elkes, Frazer, Schulman & Stewart, 1945) or in mixtures of oppositely charged inorganic colloids (Hazel & McQueen, 1933; Hazel, 1938).

Some pairs of proteins, however, do not precipitate each other in the conditions defined. Whether this is because such proteins fail to combine, or because they combine to form soluble compounds is unknown, but evidence that soluble compounds can sometimes be formed has been provided by electrophoretic studies on proteins of egg white (Longsworth, Cannan & MacInnes, 1940).

The precipitates formed between different proteins or between proteins and nucleic acid can

usually be dissolved by adding NaCl. The solution of compounds formed between tobacco mosaic virus and a number of proteins is accompanied by the splitting of the compounds into the original components. The addition of NaCl also splits compounds formed between nucleic acid and protamines or histone (Mirsky, 1943; Bang, 1904). A possible explanation of this is that anions and cations of NaCl substitute negatively and positively charged components, respectively, in the salt-like compounds.

Lack of precipitation at pH values at which components of mixtures carry electric charges of like sign may be due either to their failure to combine or to formation of soluble compounds. For some mixtures it was possible to show that there is no combination. For example, tobacco mosaic virus did not combine to any appreciable extent with globin at pH 9·0. Similarly, Longsworth & MacInnes (1941–2) gave electrophoretic evidence that ovalbumin did not combine with yeast nucleic acid and salmine outside pH ranges at which precipitation occurred, except for very short pH ranges around the isoelectric point of the albumin. Components of some mixtures, however, seem to be able to combine to some extent even at pH values at which they carry electric charges of like sign. For example, Stenhagen & Teorell (1939) found electrophoretic evidence of combination between thymonucleic acid and human serum albumin at pH values on the alkaline side of the isoelectric point of the albumin.

Suspensions of heat coagulated proteins behave in much the same way as protein solutions in that they can combine and flocculate with solutions of some oppositely charged proteins or nucleic acid, and the combinations can be split by NaCl. Also two oppositely charged protein suspensions can flocculate each other. However, suspensions of some heat coagulated proteins can behave differently from solutions of the same proteins. For example, no combination could be detected between suspensions of heat-coagulated globin and solutions of serum albumin at any pH value, whereas globin combined and precipitated with serum albumin when solutions of the two were mixed at pH values between their isoelectric points. The presence of opposite electric charges is not always sufficient to cause combination between a protein suspension and a solution of another protein.

Adsorption of solutions of proteins and nucleic acid by charcoal seems to depend on a mechanism different from that which operates when solutions of these materials combine with suspensions of heat-coagulated proteins. Whereas the former is not influenced by the sign of electric charges, the latter seems to be conditioned by it. The proportion of the material adsorbed by charcoal decreases with the increasing charge of the material, irrespective of the sign of the charge. Mutual repulsion of similarly charged molecules seems to interfere with their accumulation on the surface of charcoal. The effect of NaCl, when it causes an increase in the proportion of adsorbed protein or nucleic acid, may be due to a virtual decrease in the net charge of these materials. It is known that the presence of salts decreases electrophoretic mobility of proteins, and this is attributed to crowding of ions of opposite charge around protein molecules (Abramson, Moyer & Gorin, 1942).

It might be expected that combination between tobacco mosaic virus and ribonuclease would differ from that between the enzyme and other proteins or between the virus and other proteins, as the virus contains nucleic acid hydrolyzable by the enzyme. However, no essential difference was found between combinations of the virus with the enzyme and those of the virus with other proteins or of ribonuclease with other proteins. Mixtures of the virus with ribonuclease precipitated at pH values between the isoelectric points of the two, in the same manner as did mixtures of the virus with globulin, globin or clupein, or mixtures of ribonuclease with globulin. Isoelectric suspensions of the virus did not combine with ribonuclease or with other proteins. Some ribonuclease combined with the virus at pH 9·0, i.e. on the alkaline side of the isoelectric points of both materials, but a similar proportion of the enzyme could combine at this pH with suspensions of heat coagulated protein component of the virus and of heat-coagulated serum globulin. As pH 9·0 is not far removed from the isoelectric point of the enzyme, this result can be explained by the attraction of some virtually uncharged molecules of the enzyme by strongly charged particles of the virus or of the protein suspensions.

The virus nucleic acid in native tobacco mosaic virus is not hydrolyzed by ribonuclease until it is separated from the protein moiety of the virus (Loring, 1942). Similarly, the protein of the native virus is not susceptible to proteolytic activity of pepsin until heat denatured. Specific affinity could be demonstrated between heat-denatured virus protein and pepsin, but not between native virus and pepsin (Kleczkowski, 1944). By analogy, it seems possible that there is a specific affinity between ribonuclease and the free virus nucleic acid, though there is not between the enzyme and the intact virus. This possibility has not been tested experimentally. The only fact which could be interpreted as evidence for some specific affinity between tobacco mosaic virus and ribonuclease is that the enzyme inhibits infectivity of the virus much more efficiently than do globin or clupein. The effect of pH and of NaCl on the inhibition

caused by each of the three materials seems to indicate that their combination with the virus contributes to the inhibition, although it is possible that there are additional mechanisms involved in the process. Whenever combination of any one of the three materials with the virus had been split or prevented, either by adding NaCl or by adjusting the pH suitably, the inhibition was correspondingly reduced.

SUMMARY

1. Combinations of tobacco mosaic virus with proteins having different isoelectric points and with yeast nucleic acid were studied. Combinations of some of these proteins (ribonuclease, globin, clupein, serum albumin and serum globulin) with each other and with the nucleic acid were tested for comparison with the virus.

2. When solutions of some pairs of these materials were mixed at pH values at which they were oppositely charged they precipitated each other; most of the precipitates could be dissolved by adding NaCl. The behaviour of tobacco mosaic virus did not differ from that of other proteins. The addition of 0·3M-NaCl to mixtures of tobacco mosaic virus with clupein, globin and ribonuclease separated 20–90% of the materials previously combined.

3. Suspensions of heat-coagulated proteins behaved similarly to protein solutions; they could be flocculated by solutions of oppositely charged nucleic acid or clupein; some oppositely charged suspensions also flocculated one another. At pH values remote from their isoelectric points suspensions combined with solutions of other proteins or of nucleic acid if their charges and those of the suspensions were of opposite sign; these combinations could be split to varying extent by adding 0·3M-NaCl. There are also indications that electrically charged suspensions can attract protein molecules carrying no net charge.

4. The inhibiting effects of ribonuclease, globin and clupein on the infectivity of tobacco mosaic virus at pH 6·0 was diminished by 0·3M-NaCl. Changing the pH from 6·0 to 9·0 reduced the inhibiting effects of ribonuclease and globin, but it did not influence the effect of clupein; it also considerably reduced the amount of ribonuclease or globin combined with the virus, but had no effect on its combination with clupein.

5. No evidence was found of any specific affinity responsible for the combination of tobacco mosaic virus and ribonuclease, apart from the fact that the enzyme inhibited infectivity of the virus much more strongly than did other tested proteins, which were capable of combining with the virus.

6. Unlike the combination of nucleic acid and proteins with other proteins, the mechanism of adsorption by charcoal is not conditioned by the sign of their electric charge.

REFERENCES

Abderhalden, E. & Fodor, A. (1920). *Kolloidzschr.* **27**, 49.
Abramson, H. A., Moyer, L. S. & Gorin, M. H. (1942). *Electrophoresis of Proteins.* New York: Reinhold Publishing Corp.
Anson, M. L. & Mirsky, A. E. (1930). *J. gen. Physiol.* **13**, 469.
Bang, I. (1904). *Beitr. chem. Physiol. Path.* **4**, 115.
Bawden, F. C. & Pirie, N. W. (1937*a*). *Proc. Roy. Soc.* B, **123**, 274.
Bawden, F. C. & Pirie, N. W. (1937*b*). *Brit. J. exp. Path.* **18**, 275.
Bawden, F. C. & Pirie, N. W. (1943). *Biochem. J.* **37**, 66.
Butler, J. A. V. (1945). *Nature, Lond.*, **156**, 781.
Dow, P. (1935–6). *J. gen. Physiol.* **19**, 907.
Elkes, J. J., Frazer, A. C., Schulman, J. H. & Stewart, H. C. (1945). *Proc. Roy. Soc.* A, **184**, 102.
Hammarsten, E. (1924). *Biochem. Z.* **144**, 383.
Hazel, F. (1938). *J. Phys. Chem.* **42**, 409.
Hazel, F. & McQueen, D. M. (1933). *J. Phys. Chem.* **37**, 553.
Hitchcock, D. I. (1925). *J. gen. Physiol.* **8**, 61.
Kekwick, R. A. (1938). *Biochem. J.* **32**, 552.
Kleczkowski, A. (1941). *Brit. J. exp. Path.* **22**, 188.
Kleczkowski, A. (1943). *Biochem. J.* **37**, 30.
Kleczkowski, A. (1944). *Biochem. J.* **38**, 160.
Kleczkowski, A. (1945). *Brit. J. exp. Path.* **26**, 41.
Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
Lissitzin, M. A. & Alexandrowskaja, N. S. (1933). *Hoppe-Seyl. Z.* **21**, 156.
Longsworth, L. G., Cannan, R. K. & MacInnes, D. A. (1940). *J. Amer. Soc. chem.* **62**, 2580.
Longsworth, L. G. & MacInnes, D. A. (1941–2). *J. gen. Physiol.* **25**, 507.
Loring, H. S. (1942). *J. gen. Physiol.* **25**, 497.
Loring, H. S. & Stanley, W. M. (1937). *J. biol. Chem.* **117**, 733.
Michaelis, L. & Rona, P. (1919). *Biochem. Z.* **94**, 225.
Michaelis, L. & Szent-Györgyi, A. (1920). *Biochem. Z.* **103**, 178.
Mirsky, A. E. (1943). *Advances in Enzymology*, **3**, 1. New York.
Miyake, S. (1927). *Hoppe-Seyl. Z.* **172**, 225.
Neurath, H., Greenstein, J. P., Putnam, F. W. & Erickson, J. O. (1944). *Chem. Rev.* **34**, 157.
Osato, S. (1922). *Biochem. Z.* **132**, 485.
Przylecki, S. J. & Grynberg, M. Z. (1932). *Biochem. Z.* **251**, 248.
Rothen, A. (1940). *J. gen. Physiol.* **24**, 203.
Snell, F. D. & Snell, C. T. (1936). *Colorimetric Methods of Analysis*, **1**. London: Chapman and Hall, Ltd.
Snell, F. D. & Snell, C. T. (1937). *Colorimetric Methods of Analysis*, **2**. New York: Van Nostrand Co.
Stanley, W. M. (1934). *Phytopathology*, **24**, 1055.
Stenhagen, E. (1938). *Biochem. J.* **32**, 714.
Stenhagen, E. & Teorell, T. (1939). *Trans. Faraday Soc.* **35**, 743.
Steudel, H. & Peiser, E. (1922). *Hoppe-Seyl. Z.* **122**, 298.
Tiselius, A. (1937). *Biochem. J.* **31**, 1464.
Ugglas, B. (1914). *Biochem. Z.* **61**, 469.