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# 150. THE INACTIVATION OF SOME PLANT VIRUSES BY UREA

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(Received 30 July 1940)

## INTRODUCTION

OF the simple organic substances known to affect proteins in neutral solution, urea has been the most extensively studied. There is no obvious reason for this, for urea is neither a more powerful denaturing agent nor a better protein solvent than some of the others. The fact that it is a normal metabolic product is not really relevant, for most of the effects studied are observed only at 10 or more times the physiological concentration. Detailed studies on the action of urea have been made only in recent years, but some of its more striking effects were early noticed.

Buchner [1874; 1876] reviewed and extended earlier physiological studies on muscle stimulation, and noticed that within a few minutes of putting strips of muscle in concentrated urea solutions there was osmotic shrinking, followed by great swelling, though little of the material went into solution. Limbourg [1887] made similar experiments with frog nerves and seems to have been the first to discover that urea solutions could dissolve some otherwise insoluble proteins. He described the behaviour of such solutions towards commonly used protein precipitants and in 1889 found that solutions of fibrin in urea did not coagulate when heated. Loss of heat coagulation in 10% urea solutions was found with Bence-Jones protein by Magnus-Levy [1900] and with the commoner proteins by Ramsden [1902], who also described graphically the disintegration of a frog in saturated urea solution, and recommended the latter as a histological reagent. Braun [1933] and Stein & Miller [1938] adopted this recommendation for the histological recognition and preparation of elastin, which is one of the few proteins insoluble in concentrated urea solution. The solvent properties of urea have also been used in the preparation of other proteins, e.g. by Cook & Alsberg [1931] for glutenin, by Urban [1936] for a liver globulin mixture, by McKay & Lamson [1936] for pollen proteins and by Kondo *et al.* [1939] for soya bean proteins. Walker [1940] extracted antigens from *Salmonella aertrycke* with urea, but the method is not generally applicable, for Fuller [1938] and Miles & Pirie [1939] found it of no value with other bacteria.

Many workers have found that the presence of urea protects proteins from the precipitating effects of some reagents. Pauli & Rona [1902], Arnd & Hafner [1926], Jirgensons [1936] and Heim [1937] showed that more salt was necessary to get precipitates in solutions of gelatin, euglobulin, haemoglobin and fibrinogen if urea was present. Moser [1927] found serum proteins more difficult to precipitate by dilution or acidification in the presence of urea, and Ogiu & Pauli [1932] stabilized the complex of serum albumin and colloidal gold with urea and with glycine.

Detailed studies of the changes occurring when a protein is dissolved in strong urea solution, and the correlation of these with observations made in the presence of other organic substances, date from Spiro's [1900] work on their effect on coagulation by heat. Ramsden [1913], however, was the first to relate these changes with the group of protein-modifying processes known as denaturation. Although Ramsden's results were published in both English and German [Ramsden & Chavasse, 1913], they attracted little attention, and widespread interest was not aroused until Anson & Mirsky [1929] studied the denaturation of haemoglobin by urea and some other substances. Burk & Greenberg [1930], while continuing their earlier [1928] measurements of the osmotic pressure of proteins in 40% urea solution, described the denaturation of egg albumin and horse haemoglobin, and found that the osmotic pressure of haemoglobin denatured in this way corresponded to a molecular weight of 34,000, i.e. one-half of the original value.

Hopkins [1930] found that denaturation of egg albumin in the presence of urea proceeded more rapidly at 0° than at 37°, an interesting observation confirmed by Ramsden [1930]. Stanley & Lauffer [1939] found that the disintegration of tobacco mosaic virus also proceeded more rapidly at 0° than at 25°, but that it proceeded more rapidly at 40° than at 25°. Whether this increase in denaturation rate at low temperatures applies at all generally to proteins is uncertain, for Diebold & Jühling [1938] found that fibrinogen was rapidly denatured at 37°, whereas at 4° it was more stable in the presence of 23% urea than it was in water. However, the behaviour of fibrinogen seems to depend on its purity, as Diebold [1938] found that it was completely destroyed at 4° if urea was added to unfractionated plasma. Jühling & Wöhlisch [1938], who confirmed the difference between plasma and isolated fibrinogen, suggested that it may result from the activation of a fibrinolytic enzyme normally present in plasma in an inactive form.

### 1. *Evidence for the attachment of urea to proteins*

Observations similar to those of Buchner [1876] on the swelling of cornea in urea solutions have been made by Stoeltzner [1925] and Stoye [1925] on pieces of dura mater. Lloyd & Mariott [1933] measured the swelling of silk fibres in urea solution and found that there was a weakening of the lateral binding forces of the fibres. They suggested that urea was absorbed on the internal surfaces of the fibres, a conclusion agreeing well with the observation of Trogus & Hess [1933] that silk fibroin, after soaking in 50% urea, gave an X-ray pattern showing a regular arrangement of urea within the fibre. On the other hand, Astbury *et al.* [1935] do not mention such a pattern in washed films of denatured edestin or egg albumin prepared from urea solutions. Worschitz & Herman [1934] found that the micelle structure of muscle fibres disappeared in urea solutions and did not reappear when the urea was washed out. The phenomena of the swelling of crystalline or fibrous structures with urea and other substances were discussed by Katz [1932] and by Lloyd & Shore [1938]. Insoluble proteins that are not fibrous have been little studied, although Fischer & Sykes [1915] published some observations on the swelling of blocks of gelatin in solutions of urea too dilute to cause solution.

Attempts to use urea to measure the "bound water" in proteins have given further evidence that the first step in the action is the binding of urea to the protein. For example, Oda [1930] found that 5% of the water in serum was not available for dissolving glucose, whereas it was all available for the solution of urea. Similarly, Versmold [1931] measured the freezing points of solutions of

egg albumin containing either glucose, glycerol or urea and found that much more of the urea was bound to the protein. Finally, measurements of the optical rotatory power of proteins in different solvents put urea in a special category. Dill & Alsberg [1925] found that gliadin had a higher rotation in 30% urea than in various dilutions of ethyl and propyl alcohols, and Pauli & Weiss [1931] found that proteins in solutions of sodium benzoate and salicylate, which like urea are denaturing agents, had the same rotation as in water, whereas in urea solutions they had a higher rotation.

## 2. *The size and shape of proteins in urea solution*

Solution in urea seems to affect the osmotic pressure of individual proteins differently. Burk & Greenberg [1930] found that in urea solution horse haemoglobin had half its normal molecular weight, as measured by osmotic pressure, and edestin one-quarter. Burk [1937, 1] found similar changes in osmotic pressure with amandin and excelsin, and [1940] *Limulus* haemocyanin. Wu & Yang [1932] confirmed the fall in the molecular weights of ox and horse haemoglobins but found the values for sheep and dog haemoglobins to remain unchanged after treatment with urea, although Drabkin [1939] has shown that this treatment does denature dog haemoglobin. Hand [1935] claimed that the effects of hydration had been overlooked in these measurements on haemoglobins, but his criticism met with little support [cf. Steinhart, 1938]. Similar changes have been recorded with the muscle proteins [Weber, 1933; Weber & Stover, 1933]; in 45% urea myosin had an apparent molecular weight of  $10^5$  instead of about  $10^6$  and myogen one of 34,000 instead of 81,000. On the other hand, serum albumin, investigated in some detail by Burk [1932] and Pauli [1934], behaved differently. Burk discussed the corrections that must be applied because of departures from the ideal solution law and concluded that serum albumin had the same molecular weight in urea solution after denaturation as it had in water or in 75% glycerol. Burk [1937, 2; 1938] also showed that serum globulin and gliadin were denatured by urea without changing molecular weights. Huang & Wu [1930] denatured egg albumin in different ways and found differences between the osmotic pressures in urea solution, but Burk & Greenberg [1930] and Burk [1937, 1] found the same osmotic pressure for egg albumin whether it was dissolved in water or in 40% urea. The fact that Williams & Watson [1937] found a sedimentation constant for egg albumin in 50% urea corresponding to a molecular weight of 21,000 instead of the normal value of 36,000 at first sight contradicts Burk's results. However, all these results assume, that the particles of both native and denatured protein are spherical, and Bull [1940] has pointed out that the increase in viscosity of egg albumin on denaturation is evidence that the particles are no longer spherical. This conclusion is compatible with the observation of Lee & Wu [1932] that the area per molecule of films of native and urea-denatured egg albumin, making the usual assumptions about molecular weight and density, are 8400 and 10,400 sq. Å., but this increase in area was not confirmed by Bull [1938].

Too few observations have been made on the viscosities of native and urea-denatured proteins to permit any valid generalizations, but there appears to be an interesting difference between initially anisodimensional and viscous proteins and the more mobile proteins [cf. Hand, 1935]. Liu [1933], Neurath & Saum [1939] and Bull [1940] found a rise in the relative viscosity of the globular egg albumin on denaturation, whereas Frampton [1939] and Edsall & Mehl [1940] found that denaturation increased the mobility of the rod-shaped proteins

tobacco mosaic virus and myosin. Many workers have noticed the increased fluidity given by urea to glutinous products, and this property was used by Dold [1924] for liquefying sputum as a preliminary to examination for tubercle bacteria.

### 3. *Evidence on the nature and reversibility of the changes wrought by urea*

It is clear that the action of urea on a protein is complex and that the strength of the urea, the time of action and the temperature may determine which of the possible actions occurs. The milder changes, such as swelling, increase in solubility and permeability, and perhaps the attachment of urea to parts of the protein, are generally reversible [Heim, 1937]. Carpenter & Lovelace [1938], from a study of the rotatory dispersion, found with gelatin that the effects even of concentrated urea were completely reversible, but this is exceptional and with most proteins intense treatments cause a change that may properly be regarded as denaturation [cf. Mirsky & Pauling, 1936]. This change is again complex and proceeds through a succession of stages according to the severity of the treatment with urea. For example, Beck & Schormüller [1937] in studying the solution of horse meat in urea found that heating solutions of the already denatured protein had a further effect in preventing its precipitation on dialysis. Diebold and Juhling [1938] obtained similar results with fibrinogen, and Steinhardt [1938] found that only 30% of a sample of horse haemoglobin precipitated on dialysis after exposure to 4*M* urea whereas all precipitated after exposure to 7.46*M*. Edsall & Mehl [1940], extending the observation of von Muralt & Edsall [1930] on the loss of anisotropy of flow of myosin in strong urea solutions, commented on the fact that this loss occurred when the treatment was insufficient to reduce the viscosity to its minimum value.

Hopkins [1930] first showed that proteins varied in their resistance to denaturation by urea. The denatured protein is usually recognized by diluting or dialysing solutions until the urea is too dilute to keep it in solution. Steinhardt [1938] and Burk [1937, 2] have stressed the uncertainty of this criterion, partly because of the solubility of denatured protein in the presence of native protein and partly because of the readiness with which the first stages of denaturation may be reversed by dialysis. Steinhardt also presented evidence that denaturation of some proteins did not occur in the urea, even though dissociation had taken place, but that it was a result of subsequent treatments such as dilution or dialysis. On the other hand, Burk [1932] found that the heat coagulation of serum albumin could be partly reversed by dissolving it in urea and dialysing at a low temperature; Laporta [1932] made similar claims for other proteins.

For obvious reasons experiments on proteins with measurable specific activities would give the most satisfactory evidence that urea has had an effect that is irreversible by dilution or dialysis. As yet few of these have been made. The concentrations of urea used in experiments with pepsin and trypsin have not affected them irreversibly. The behaviour of fibrinogen is interesting, for Wöhlisch & Kiesgen [1936] found that in 30% urea it could no longer be coagulated by heat, alcohol or thrombin, whereas Meissner & Wöhlisch [1937] recovered from the solution fibrinogen that could be clotted. Diebold & Jühling [1938] examined the system in greater detail, finding that thrombin could act in the presence of 15% urea but that the precipitation of fibrin was prevented. A water-soluble protein that could no longer be clotted with thrombin could be made by more vigorous treatment with urea. The effects of urea on the activities of viruses and bacteria are described later in this paper.

Steinhardt's [1938] hypothesis that urea and other amides act by competing with peptide bonds in one part of a protein for association with neighbouring bonds in the protein grid is compatible with the evidence so far adduced. On this hypothesis the protein is held together by these associations and the probability that a protein will return to its original configuration on the removal of urea will depend on the number of these associations that have been broken and have to reform. With conjugated proteins, urea may split off the prosthetic group. Experience shows that this usually leads to greater instability of the protein moiety, so that denaturation of that might also be expected. Some agents, e.g. sodium dodecyl sulphate [Sreenivasaya & Pirie, 1938] may dissociate a prosthetic group without denaturing the protein, but no example is known of urea acting in this way, although it is presumably possible. On the other hand, examples of denaturation without the liberation of the prosthetic group are known.

The most characteristic chemical difference between native and denatured proteins is the presence in the latter of free —SH groups, or of groups that can readily be turned into —SH groups by reducing agents. This effect was first demonstrated by Hopkins [1930] with egg albumin and horse and sheep serum proteins after denaturation by urea. Burk [1937, 1] confirmed these results and showed that amandin, edestin, excelsin, sheep haemoglobin and myogen also gave —SH groups, but not gliadin, zein or pepsin. Quantitative measurements of the —SH groups formed by action of urea and related substances have been made by Greenstein [1938; 1939] on egg albumin and by Greenstein & Edsall [1940] on myosin. Not all the available —SH was liberated from myosin by prolonged treatment with urea, for on denaturation with guanidine twice the apparent cysteine content was found. Tobacco mosaic virus also gives an —SH reaction after denaturation with urea. Little other chemical work has been done on differences between native and urea-denatured proteins. Chou & Wu [1936] found that it had no effect on the formaldehyde titrations of five proteins, suggesting that the —NH<sub>2</sub> groups were not concerned in the changes, although Hopkins [1930] and Wu *et al.* [1931] noticed a change in pH on denaturation.

#### 4. *The effects of urea on bacteria and tissues*

We have already referred to the first experiments on tissues in which urea was used as a solvent, and we conclude this survey by describing some systems in which it is probable, though not certain, that the changes accompanying exposure to urea result from its action on protein. Mustard and cress seeds exposed to 5% urea did not germinate [Ramsden, 1902]; *Esch. coli* barely grew in media containing 8% urea [Wilson, 1906] and was more easily lysed by its bacteriophage in the presence of 5% urea [Bronfenbrenner & Hetler, 1933]. Changes to filamentous forms were described by Wilson [1906] and by Péju & Rajat [1906] when some bacteria were grown on media containing 2–5% urea, but many bacteria, e.g. staphylococci, streptococci and sarcinae, were apparently unaffected. Symmers & Kirk [1915] measured the rate at which *Ps. pyocyanea* was killed by the presence of urea when suspended in blood, and found 25 g. per 100 ml. to be the lowest effective concentration. They recommend the liberal use of urea in the treatment of wounds and mention the use of solutions as mouth washes for diphtheria carriers and for sterilizing tuberculous sputum. Individual bacteria differ widely in their resistance to concentrated urea solutions [Dold, 1924; Foulger & Foshay, 1935; Finger, 1937]; *Mycobacterium tuberculosis* was the most resistant tested and was killed by 3 hr. exposure to saturated urea at 37°.

Bacterial spores were much more resistant than bacteria. Rooschütz [1935] noted their presence in most commercial samples of urea, and Dold & Weyrauch [1924], having found spores to be viable after a month's exposure to saturated urea at room temperature, recommended the use of urea solutions in their isolation.

In popular medicine the use of urine and of plant extracts containing related substances such as allantoin, e.g. comfrey [Macalister, 1912], for promoting wound healing is some thousands of years old. Solid urea was used to prevent the suppurating of wounds by Symmers & Kirk [1915], but no further use of the treatment seems to have been made until Millar [1933] described its beneficial effects on sloughing cancers. Since then the successful use of solid urea on a large number of wound cases has been described by Holder & MacKay [1937; 1939], by Muldavin & Holtzmann [1938] and others. Strong solutions seem to be equally effective against chronic infections of the ear, nose and throat [Foulger & Foshay, 1935; Mertins, 1937]. Treatment with solid urea resembles in some ways the well-known treatment of wounds with fly maggots. Presumably part of the benefit in both treatments comes from the removal of damaged tissue, by solution with urea and by selective feeding with the maggots. It is unknown why urea does not dissolve healthy tissues, but this relatively specific action has been noted by Stoeltzner [1925] and Stoye [1925], who claimed that the injection of concentrated urea solutions under non-adherent scar tissue led to its ready removal and to the smooth healing of the wound. They found, however, that adhering scar tissue was apt to become necrotic with this treatment. Tissues are readily permeable to urea, and it may be that where there is an undamaged blood supply, a sufficiently high concentration of urea to dissolve tissue proteins cannot be maintained. In wound healing, bacteriostatic action is doubtless of great importance, but it has also been suggested, notably by Robinson [1938], that urea has a directly stimulating action on cell proliferation. Mond & Hoffman [1928] found that urea as dilute as 0.3 *M* damaged the surface of red blood corpuscles and tended to make lysis easy. Smadel *et al.* [1938] have also made similar observations, but this property does not seem to have caused any difficulties in the clinical use of urea.

##### 5. *The effect of urea on viruses*

Burnet [1933] tested the resistance of 24 different dysentery coli bacteriophages to urea and divided them into three groups according to whether they were rapidly inactivated, slowly inactivated or unaffected by 27.7% urea; he suggested that the smaller phages were the more resistant. McKay & Schroeder [1936] found that rabies and anterior poliomyelitis viruses lost both their ability to infect and to immunize rabbits after exposure to 40% urea. Hoyt & Warner [1940] confirmed the inactivation of rabies virus, but found that after less severe treatment, so that it was not completely inactivated, it retained its immunizing power. Smadel *et al.* [1938] found that in 22% urea there was an initial rise in the sedimentation constant of vaccine virus followed by a fall, whereas in 40% the fall was continuous. Inactivation was complete in 3 days in 22% urea, whereas a 10–15% solution was without any apparent effect. These changes presumably correspond to the swelling that has been observed with some other proteins in dilute urea and with the denaturation that occurs in concentrated solutions. Höring [1939] showed that concentrated urea quickly inactivated yellow fever virus, whereas in dilute urea the virus was about as stable as it was in water.

Bawden & Pirie [1937] found that three strains of tobacco mosaic virus were more resistant to urea than were the proteins used by Hopkins [1930] or than some of the phages used by Burnet [1933]. No details were given, though the denaturation of the viruses and the loss of liquid crystallinity after several hours' exposure to saturated urea were mentioned. Mehl [1938] also mentioned the loss of anisotropy of flow of tobacco mosaic virus preparations after treatment with urea. Frampton & Saum [1939] and Frampton [1939] reported that solution of tobacco mosaic virus in 6 *M* urea and 0.1 *M* phosphate buffer caused a hundredfold increase in the diffusion constant with no change in infectivity. They interpreted this result as indicating that the urea caused the virus particles to disaggregate into single molecules of molecular weight around 100,000. They suggested that these were the true virus molecules and that the large particles with weights equivalent to molecular weights of many millions were merely aggregates. However, no increase in infectivity accompanied the urea treatment such as would be expected from such a disaggregation into small infective units, and later work has not confirmed this view. Stanley & Lauffer [1939] were unable to obtain any evidence that the low molecular weight protein produced by the action of urea was infective. They found that the disintegration depended on the salt content, pH and temperature, and any residual activity in treated virus preparations was always found to be associated with remaining protein of high molecular weight. Measurements in the ultracentrifuge [Martin, 1939] indicated that the inactivated virus had a molecular weight of 400,000, but osmotic pressure measurements [Stanley & Lauffer, 1939] showed that on continued action of urea it fell to 40,000.

## EXPERIMENTAL

### 1. *Materials and methods*

In this paper experiments are described with the four viruses, tobacco mosaic, tomato bushy stunt, potato "X" and tobacco necrosis. The virus preparations used were made by the methods described previously [Bawden & Pirie, 1937; 1938, 1; 1938, 2; Pirie *et al.* 1938]. The tobacco necrosis virus used probably differs from that used earlier, for although the preparations used were made by the methods described then we have been unable to get any crystalline material. Instead, the whole preparations have consisted of amorphous material closely resembling the amorphous two-thirds of the previous preparations. The serological reactions of our preparations also differ from those of the earlier preparations. It seems probable that the virus culture used in 1938 was a mixture of strains, of which only one was crystallizable, and that in repeated transfers during the last two years this strain has been lost.

Because of shortage of material only a few tests were made with potato virus "X" and tobacco necrosis virus. Most were made with tobacco mosaic virus, because it is both readily obtained in large quantities and shows the convenient property of anisotropy of flow. Qualitative observations on the loss or diminution of this property, made by shaking solutions in test tubes, 0.5-1 cm. in diameter, between crossed polarizers, give valuable preliminary indications of the severity of treatment necessary to disintegrate the virus. This provides quite a sensitive test, for the disintegration products not only themselves fail to show anisotropy of flow but also impede the orientation of the still undamaged virus so that it will no longer form a liquid crystalline layer and so that a greater rate of shear is necessary to get maximum orientation. Some treatments destroy the infectivity of tobacco mosaic virus without disintegrating

it. These treatments also leave the anisotropy of flow and serological reactions unimpaired, but urea has not been found to do this.

For simplicity the purified materials used in our tests will be called viruses, although their exact relationships with the viruses as they occur in infective saps is still uncertain. With tobacco mosaic virus and potato virus "X" it is known that purification by the methods used leads to a reduction in infectivity and to some other changes, which have been attributed to the linear aggregation of virus particles [Bawden & Pirie, 1937; 1938, 1, 2, 3]. Except for tobacco necrosis virus, which was tested only for changes in infectivity, the treated virus preparations were tested both for their infectivity and serological activity. In the infectivity tests dilutions were made in 0.1 *M* phosphate buffer at pH 7, and local lesion counts were made at two dilutions of the inocula, usually at  $10^{-4}$  and  $10^{-5}$  g. protein per ml. The host used for lesion counts for tobacco mosaic and tomato bushy stunt viruses was *Nicotiana glutinosa*, for potato virus "X" *N. tabacum*, var. White Burley, and for tobacco necrosis virus *Phaseolus vulgaris*, var. Canadian Wonder. To reduce to a minimum the errors arising from differences in the susceptibility of individual plants, infectivity tests were all arranged in the form of Latin squares or incomplete blocks [Youden, 1937]. In each test at least six leaves were inoculated with each dilution of the preparation tested. As solutions containing the same quantities of active virus can often give widely different numbers of local lesions if they contain different amounts of salts or other materials, the compositions of all test and control inocula were adjusted to be at the same pH, and to contain the same amounts of urea, salts and protein before inoculation.

The dilutions in the serological tests were made in 0.85% NaCl solution. 1 ml. of antiserum at a dilution of 1/50 was added to a series of tubes each containing 1 ml. of virus solution at different concentrations, and the smallest amount of virus in g. to give a precipitate visible to the eye in 2 ml. of such mixtures is recorded as the serological titre. The serological titres of different preparations of tomato bushy stunt virus are remarkably constant, but different preparations of tobacco mosaic virus and of potato virus "X" give widely different titres. The titre of any one preparation remains constant and reproducible, changes in it accurately reflecting changes in the virus. Therefore, as the tests described were carried out over a long period and with different virus preparations, although the serological titres given in any one table are strictly comparable, they cannot necessarily be compared with those in the other tables or with those previously published.

## 2. Effect of different concentrations of urea

It is well known that the rate at which a disinfectant works is a non-linear function of its concentration [Smith, 1921], and that below a critical concentration it has no appreciable effect. This is also true for the killing of bacteria by urea, for which Wilson [1906] and Symmers & Kirk [1915] have found the critical range of concentration to be from 8 to 25%, i.e. from 1.33 to 4.17 *M*. Tables 1-4 show the effects of variations in the urea concentration on the rate of inactivation of the four viruses. They show clearly that concentrated urea has had an effect on the specific activities of all the viruses that is not reversed by dilution. They also show that the individual viruses differ widely in their resistance to the inactivating action of urea, and that, as with bacteria, there is for each one a critical concentration below which there is little or no inactivation. With all the viruses, the reduction in infectivity is approximately proportional to the reduction in serological activity, and with none is there any indication

that the proteins with low molecular weights produced by the action of urea on the large virus particles have any specific virus activities as suggested by Frampton & Saum [1939] for tobacco mosaic virus. In establishing the lack of action, or more probably the reversibility of the action, of dilute urea on the viruses the results also show that it is legitimate to study the kinetics of the action by diluting the mixtures largely when the action is to be stopped.

Table 1. *Effect of concentration of urea on inactivation of tobacco mosaic virus*

Urea <i>M</i>	ml. of phosphate and urea mixed to get molarity		ml. of phosphate and urea added in diluting		Serological titre	Average no. of lesions at	
	Phosphate	Urea	Phosphate	Urea		10 <sup>-4</sup>	10 <sup>-5</sup>
8	0.0	0.8	2.0	0.0	1/5000	1	0
7	0.1	0.7	1.9	0.1	1/10,000	2	0.5
6	0.2	0.6	1.8	0.2	1/40,000	15	3
5	0.3	0.5	1.7	0.3	1/80,000	24	5
4	0.4	0.4	1.6	0.4	1/320,000	65	28
3	0.5	0.3	1.5	0.5	1/640,000	116	44
	Control; urea diluted before adding virus				1/640,000	117	54

To 0.2 ml. of 1.64% tobacco mosaic virus was added sufficient *M*/10 phosphate at pH 7.1 and 10 *M* urea solution to give 1 ml. of solution at the required molarity. After 20 hr. at 15° each was diluted with phosphate and urea to a final volume of 3 ml., i.e. to 2.67 *M* urea, and tests were made 36 hr. later.

Table 2. *Effect of concentration of urea on inactivation of tomato bushy stunt virus*

Urea <i>M</i>	Serological titre	Average no. of lesions per leaf at	
		10 <sup>-4</sup>	10 <sup>-5</sup>
8	No ppt. at 1/5000	0	0
7	1/10,000	12	6
6	1/160,000	108	47
5	1/640,000	137	82
Control	1/640,000	157	79

The samples were prepared as in Table 1 and were exposed for 20 hr. at pH 8 and 15°. They were then diluted till the urea was 1.6 *M* and tested 36 hr. later.

Table 3. *Effect of concentration of urea on inactivation of potato virus "X"*

Urea <i>M</i>	Time	Serological titre	Average no. of lesions per leaf at	
			10 <sup>-4</sup>	10 <sup>-5</sup>
7	10 min.*	No ppt. at 1/10,000	0	0
6	1 hr.*	No ppt. at 1/10,000	1	0
4.5	1 hr.*	No ppt. at 1/10,000	6	1
3	1 hr.*	1/40,000	63	15
1.2	36 hr.	1/320,000	133	110
	Control	1/640,000	220	190

\* These preparations spent an additional 36 hr. exposed to 1.2 *M* urea.

In all tests 0.2 ml. lots of 0.48% salt-free potato virus "X" were diluted with the volumes of water and 10 *M* urea in 0.1 *M* phosphate buffer at pH 7.1 required to give 0.5 ml. at the required molarity of urea. After the stated time at 17° they were diluted to 1.2 *M* urea and tested 36 hr. later.

Table 4. *Effect of concentration of urea on the inactivation of tobacco necrosis virus*

Urea <i>M</i>	Average no. of lesions per leaf at	
	10 <sup>-4</sup>	10 <sup>-5</sup>
7.5	0	0
5.5	10	2
3.5	82	20
Control	240	130

The samples were exposed for 20 hr. at pH 7 and 16°.

### 3. *Effect of temperature on the rate of inactivation*

The fact that proteins have a large temperature coefficient of denaturation makes Hopkins's [1930] discovery that the denaturation of egg albumin in the presence of urea proceeds faster at 0° than at 37° exceptionally interesting. Stanley & Lauffer [1939] found that the disintegration of tobacco mosaic virus in urea proceeded more rapidly at 0° than at 25°, but that it proceeded more rapidly at 40° than at 25°. We have confirmed this result with tobacco mosaic virus, and have found that potato virus "X" and tomato bushy stunt and tobacco necrosis viruses also have temperature ranges in which cooling leads to an increase in the rate of denaturation by urea. Table 5 shows the results of an

Table 5. *Effect of temperature on the denaturation of tobacco mosaic virus by urea*

Temperature	Serological titre	Average no. of lesions per leaf at	
		10 <sup>-4</sup>	10 <sup>-5</sup>
0°	1/100,000	13	4
9.5	1/400,000	20	6
20	1/800,000	31	7
37	1/200,000	6	2
55	No ppt. at 1/50,000	0	0
Control	1/1,600,000	40	12

Each sample, except the control, was exposed to 6*M* urea for 4 hr. in the presence of *M*/30 phosphate buffer at pH 7.0 and the temperature stated, they were then diluted for testing. The control was diluted immediately after mixing at 15°.

experiment on the denaturation of tobacco mosaic virus by urea between 0° and 55°, and Table 6 those for a more detailed experiment between -10° and +11°. Results of experiments at different temperatures with potato virus "X" and tomato bushy stunt virus are given in Tables 7 and 8.

The viruses used in these temperature tests vary widely in their reactions to freezing and drying, presumably because they hold water in different ways. Bushy stunt virus in salt-free solution is denatured and inactivated by freezing and thawing, whereas tobacco mosaic virus and potato virus "X" are not, and bushy stunt virus and potato virus "X" are completely inactivated when neutral solutions are dried, whereas tobacco mosaic virus is only partially inactivated. In spite of these differences in the properties of the individual viruses, the denaturation of all of them by the urea shows a similar increase in rate at low temperatures. The remote possibility exists that exposure of virus solutions to low temperatures alone might have some effect; for example, exposure to -10° might inactivate tobacco mosaic virus even though freezing at

Table 6. *Denaturation of tobacco mosaic virus by urea between -10° and 11°*

Urea <i>M</i>	Time of exposure hr.	Temperature	Serological titre	Average no. of lesions per leaf at	
				10 <sup>-4</sup>	10 <sup>-5</sup>
6	24	11°	1/50,000	3.1	0.8
		0	1/20,000	2	0.2
		-5	1/10,000	0.5	0.1
		-10	No ppt. at 1/5000	0	0
	48	11	1/40,000	5.2	0.6
		0	1/10,000	0.7	0
		-5	1/5000	0.1	0
		-10	No ppt. at 1/5000	0	0
5	24	11	1/200,000	30.7	6.6
		0	1/100,000	18.4	2.5
		-5	1/20,000	3.6	0.4
		-10	1/8000	0.8	0.2
	48	11	1/100,000	14.1	0.5
		0	1/80,000	8.5	1.4
		-5	1/20,000	6.0	0.5
		-10	1/5000	1.7	0.1
	Control; urea diluted before adding virus	1/800,000	67.1	32.3	

0.2 ml. samples of a 1.64% solution of tobacco mosaic virus were added to 0.8 ml. of 7.5 or 6.25 *M* solutions of urea in *M*/30 phosphate buffer at pH 7 and already at the temperature stated. After the stated time at the given temperatures, those exposed to 6 *M* urea were diluted with 2 ml. of water and those exposed to 5 *M* with 1.9 ml. of water and 0.12 ml. of 10 *M* urea. After 36 hr. at room temperature they were diluted further for testing.

Table 7. *Effect of temperature on the inactivation of potato virus "X" by urea*

Urea <i>M</i>	Temperature	Serological titre	Average no.
			of lesions per leaf at 10 <sup>-4</sup>
3	37°	1/40,000	15
	10	1/80,000	25
	-10	1/20,000	10
2	37	1/160,000	43
	10	1/320,000	101
	-10	1/30,000	12
	Control; urea diluted before adding virus	1/640,000	176

Samples of 0.24% solution of potato virus "X" exposed in *M*/30 pH 7.0 phosphate buffer at the temperatures stated for 1 hr. to the stated concentration of urea. Samples were then diluted to 0.5 *M* urea and left for 36 hr. at room temperature before testing.

Table 8. *Effect of temperature on inactivation of tomato bushy stunt virus by urea*

Temperature	Serological titre	Average no. of lesions per leaf at		
		10 <sup>-4</sup>	10 <sup>-5</sup>	
37°	1/160,000	71	30	
20	1/320,000	135	58	
0	1/80,000	35	11	
-12	1/20,000	0	0	
	Control; urea diluted before adding virus	1/320,000	145	80

Samples were exposed for 7 hr. in 6 *M* urea at pH 7.9 at the stated temperatures. They were then diluted to 2 *M* urea and left for 36 hr. at room temperature before testing.

this temperature does not. To test this possibility eight samples of 0.47% tobacco mosaic virus solution in pH 6.9 phosphate:borate buffer mixture [Koltzoff, 1925] were mixed with 2 vol. of either 6M urea, 6M acetamide, 6M formamide or 6M alcohol. One of each pair of these mixtures was kept at  $-10^{\circ}$  and the other at  $16^{\circ}$ . All were then diluted until the urea or other substance was 1.5M, and they were tested 36 hr. later. The serological titres of the two samples containing urea were 1/10,000 for that kept at  $-10^{\circ}$  and 1/640,000 for that kept at  $16^{\circ}$ , whereas all the other samples gave a titre of 1/2,560,000. Similarly, the effect of freezing solutions of tomato bushy stunt virus is a direct effect of the freezing and not one of the low temperature, for when infected leaves, infective sap or solutions of purified virus containing salts, are cooled below  $0^{\circ}$  the virus is not inactivated. The increased rate of denaturation at low temperatures seems to be an effect specific to urea, for other denaturants have been tested over a range in temperature and none has been found that inactivates more rapidly at  $-10^{\circ}$  than at higher temperatures.

#### 4. Effect of pH changes on the rate of inactivation

Hopkins [1930] showed that egg albumin was denatured in the pH range 5-6.5, but he made no detailed tests on the rate of denaturation by urea at different hydrogen ion concentrations. Stanley & Lauffer [1939] found that there was little or no denaturation of tobacco mosaic virus by 6M urea at pH 5.5, that it was more rapid at pH 8.2 than at pH 7.4 and that it was much slower at pH 6.4 than at pH 7.4. We have confirmed the effect of alkali in increasing the rate of inactivation of tobacco mosaic virus by urea, and have found tomato bushy stunt virus to behave similarly.

The pH of a buffer solution in the presence of concentrated urea is a matter of some uncertainty. Burk & Greenberg [1930] found that in 6.66M urea the apparent pK of acetic acid shifted from 4.6 to 5.2 and that of phosphoric acid from 6.8 to 7.2. Hopkins [1930] noticed an increase of 0.8 to 1.0 units in the pH of protein solutions on the addition of urea. There are probably at least three causes of this effect: (1) the pK shift noticed by Burk & Greenberg, (2) the pH shift of protein solutions when denatured [Wu *et al.* 1931] and (3) the presence of ammonium salts in most samples of urea and the gradual production of more of these on standing, especially if the solution is heated [Lewis & Burrows, 1912; Ogiu & Pauli, 1932; Beck & Schormüller, 1937]. In our experiments two methods were adopted for measuring the pH. In the first, 10M urea in M/10 phosphate buffer was added in suitable quantities to the virus solutions and the pH recorded by the hydrogen electrode after the mixture was diluted 10-fold (the apparent pH before such dilution was about 0.2 units higher). In the second, a virus:buffer mixture of the required pH value was mixed with a neutral solution of urea.

Table 9 shows the results of one experiment on the effect of pH on the inactivation of tobacco mosaic virus by urea. The critical range of pH within which the rate of inactivation of tomato bushy stunt virus increases rapidly is even narrower than that with tobacco mosaic virus, and is shown in Table 10. In this table, and in Table 8, it appears at first sight that urea treatment is destroying infectivity without proportionally affecting the serological activity. However, we have already shown [Bawden & Pirie, 1938, 2] that this is one of the effects of treating bushy stunt virus with alkali alone, although then it occurs only at a higher pH. Thus it seems that in the presence of urea this type of inactivation can occur in less alkaline solutions, but the effect is partially masked by secondary reactions that destroy both infectivity and serological activity.

Table 9. *Effect of changes in pH on the inactivation of tobacco mosaic virus in the presence of urea*

Urea <i>M</i>	pH	Serological titre	Average no. of lesions per leaf at	
			10 <sup>-4</sup>	10 <sup>-5</sup>
6	5.16	1/2,560,000	220	98
6	6.07	1/1,280,000	160	48
6	6.76	1/160,000	50	8
6	7.87	No ppt. at 1/1000	0	0
6	9.16	No ppt. at 1/1000	0	0
4	9.16	1/4000	3	1
0	9.16	1/64,000	180	80
Control; urea diluted before mixing		1/2,560,000	260	140

The samples were exposed for 20 hr. at 15°. 0.1 ml. of a 3.3% solution of tobacco mosaic virus solution was mixed with 0.3 ml. of a mixture of *M*/10 phosphate and *M*/20 borate made up according to Kolthoff [1925] to the pH stated. 0.6 ml. of 10 *M* urea was added to the top 5 samples, 0.4 ml. plus 0.2 ml. of water to sample 6 and 0.6 ml. of water to the last sample. After 20 hr. the requisite amount of borate or phosphate was added to each sample to bring the pH to 6.76, and water and urea were added to the last 2 samples to bring the volume to 3.35 ml. and urea concentration to 1.79 *M*. Tests were made 36 hr. later.

Table 10. *Effect of pH on the inactivation of tomato bushy stunt virus in the presence of 7.7 *M* urea*

pH	Serological titre	Average no. of lesions per leaf at	
		10 <sup>-4</sup>	10 <sup>-5</sup>
7.0	1/320,000	71	16
8.1	1/40,000	0	0
9.16	No ppt. at 1/10,000	0	0
Control; urea diluted before adding virus	1/320,000	55	15

0.15 ml. lots of Kolthoff buffer at the pH stated and containing 1 mg. of bushy stunt virus were mixed with 0.5 ml. of neutral 10 *M* urea. After 80 min. at 18.5° 2 ml. of water and 0.15 ml. of *M* acetate buffer at pH 4 were added, and tests were made after 36 hr.

##### 5. Comparison of purified virus with clarified infective sap

The experiments already described were made with purified viruses prepared by precipitation methods. It is known with potato virus "X" and tobacco mosaic virus that these methods cause a fall in infectivity and filterability, and we have suggested [Bawden & Pirie, 1937] that this arises from an aggregation of the virus into particles of a greater length/width ratio. The most plausible explanation for this is that the virus particles as they occur in the plants have materials at their ends which keep them from aggregating, and that during the purification processes these end materials are removed. In view of the known differences between tobacco mosaic virus in infective sap and in the purified state, Martin's [1939] statement that the former is more readily inactivated by urea is of some interest. Martin has published no details of his experiments and we have therefore been unable to duplicate them, but in the tests we have made comparing the behaviours of crude and purified preparations the increased stability of the latter is only slight.

Leaves from infected tobacco plants were frozen and minced, and the sap expressed through muslin and centrifuged. The pH of the supernatant fluid,

measured by the hydrogen electrode, was 5.9. A 4% solution of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  was made with this sap, which, after centrifuging off the precipitate formed, had pH 6.9. As infective sap usually contains 1–2 g. of virus per litre, a comparison solution was made containing 1.57 g. of purified virus per litre in  $M/10$  phosphate buffer at pH 6.9. Both solutions were then made up to 6M urea by the addition of solid urea, and after the required times at 15° samples were taken and diluted to 2M urea for testing later. To obtain 100 ml. of 6M urea, 73.5 ml. of solution were added to 36 g. of urea, so that the final concentration of purified virus in the mixtures was  $1.57 \times 0.735$ , or 1.15 g. per litre. To the control solutions only sufficient urea was added to give a concentration of 2M. Table 11 gives the

Table 11. Comparison of the rates of inactivation of purified and crude tobacco mosaic virus preparations at pH 6.9 in 6M urea

Time	Phosphate: infective sap mixture Serological titre	0.115% solution of purified virus in phosphate buffer					
		Lesions		Serological titre	Lesions		
		10 <sup>-4</sup>	10 <sup>-5</sup>		10 <sup>-4</sup>	10 <sup>-5</sup>	
30 min.	1/81	43	14	1/243	144	64	
2.5 hr.	1/27	6	1.5	1/27	36	14	
20 hr.	0	0	0	1/3	3	0	
Control	1/729	215	158	1/2187	204	132	

Description in text.

results of this comparison. It again illustrates the greater infectivity for a given serological titre that is characteristic of unpurified virus. It also shows that the inactivation of the virus in infective sap proceeds somewhat faster and farther than that of the purified virus, but the difference is slight and may well be caused by differences in the salt contents of the two preparations and by the action of the urea on the salts. Alternatively, it is possible that at the same time as the urea is denaturing the purified virus it also dissociates the aggregates formed during the purification processes, and that this slight increase in the number of infective units partly masks the inactivating effect and accounts for the slower inactivation of the purified virus.

#### 6. Chemical and physical changes accompanying inactivation by urea

No detailed study has been made of the changes taking place during the inactivation of the viruses by urea, but the two anisotropic viruses with low nucleic acid content behave differently from the two isotropic viruses with higher nucleic acid contents. Stanley & Lauffer [1939] found that the inactivation of tobacco mosaic virus by urea led to a separation of the nucleic acid from the protein moiety. We have confirmed this, and find that nucleic acid is also liberated from potato virus "X" during the inactivation. On the other hand, the precipitates that separate from preparations of tomato bushy stunt and tobacco necrosis viruses denatured by urea still contain nucleic acid.

The solvent action of urea on native and denatured proteins was described in the introduction. The interrelation of these two actions with fibrinogen was studied by Diebold & Jühling [1938], who found that after a few hours at 37° solutions in 0.9% NaCl had a minimum opacity when they contained 12% of urea and a maximum when they contained 20%. Below 12% the lyotropic effect of urea on native fibrinogen was being measured and above 20% its solvent action on denatured fibrinogen, while between these two concentrations

denaturation and partial precipitation were proceeding. In their behaviour towards urea bushy stunt and tobacco necrosis viruses to some extent resemble fibrinogen. Tomato bushy stunt virus is almost completely inactivated by exposure to 7 *M* urea for 20 hr. at pH 8 and room temperature, but at this urea concentration, in the presence of a trace of salt, the denatured protein is insoluble, and it dissolves only slowly in 8–9 *M* urea. On the addition of a trace of salt to solutions exposed for 10–20 hr. to 6–8 *M* urea there is an immediate, apparently irreversible precipitation. These precipitates, like the original virus, contain 5% of carbohydrate as estimated by heating with orcin and sulphuric acid. The carbohydrate can be removed from the denatured protein by extraction with boiling dilute ammonium hydroxide, as it can from virus denatured by heating or drying [Bawden & Pirie, 1938, 2]. Tobacco necrosis virus behaves similarly but the effect is more easily demonstrated as inactivation occurs with more dilute urea. The precipitate of denatured virus is also more readily soluble in urea, so that there is an immediate disappearance of the faint opalescence characteristic of the virus preparation when enough urea is added to a 0.12% virus solution to make it 8 *M*. In more dilute urea solutions, from 4 to 6.5 *M*, there is rapid precipitation, but after some hours the precipitated material dissolves. On diluting any of these solutions of inactivated tobacco necrosis virus with 0.9% NaCl solution a precipitate separates that still contains nucleic acid.

The absorption spectra of the more highly purified virus preparations have all shown a characteristic maximum at about 260  $m\mu$ , i.e. in the region associated with absorption by nucleic acid. As part of a general examination of the manner of linkage of the nucleic acid to the protein moiety we have compared the absorption spectra of the separated protein and nucleic acid with that of the intact virus. These experiments have shown that processes leading to separation of the nucleic acid lead to diminution of the absorption in the ultra-violet. This result is not unexpected, for it is well known that the intensities, and even the position of, the absorption maxima of purines and pyrimidines are affected by substitution or by changes in ionization [cf. Ellinger, 1938]. Furthermore, the treatments effecting the separation increase the clarity of the fluids, presumably because the average molecular weights of the dissolved materials are less, and this reduces the amount of light scattered, especially in the ultra-violet range. It is reasonable to suppose that much of the apparent absorption by plant virus preparations in the ultra-violet is caused by scattering. Lavin *et al.* [1939] found that tobacco mosaic virus preparations made by precipitation with ammonium sulphate absorbed more strongly than those made by ultra-centrifuging, and, as the former are the more highly aggregated, part at least of this effect may be caused by the difference in scattering.

Urea dissociation of potato virus "X" illustrates the effect on the absorption spectrum of the separation of the nucleic acid from the protein and the breakdown of the protein into smaller particles. When we described the purification of virus "X" [Bawden & Pirie, 1938, 1] we did not publish a spectrum, but in a later paper [Bawden & Pirie, 1939] preparations were described that had no absorption minimum at 245  $m\mu$ . By repeated differential high speed centrifuging of chemically purified products we now get preparations with a minimum at 245  $m\mu$ , although it is not so pronounced as those found with tobacco mosaic, tomato bushy stunt or tobacco necrosis viruses. This agrees with the figures published by Lavin *et al.* [1939]. The curves in Fig. 1 give the density of a 2 cm. layer of a 0.029% solution of potato virus "X" in the presence of 0.77 *M* urea and 0.08 *M* phosphate buffer at pH 7. For the upper curve, A, the virus was added to urea at 0.77 *M* and the spectrum taken immediately. For the lower

curve, *B*, the solution was made by adding the virus to a concentrated urea : phosphate mixture so that it was exposed to 7.1 *M* urea. After 40 min., sufficient time for complete inactivation, the mixture was diluted to 0.77 *M*. Short exposure to 0.77 *M* urea apparently had no effect on the virus, for the curve *A* was identical with that given by the virus dissolved in water.

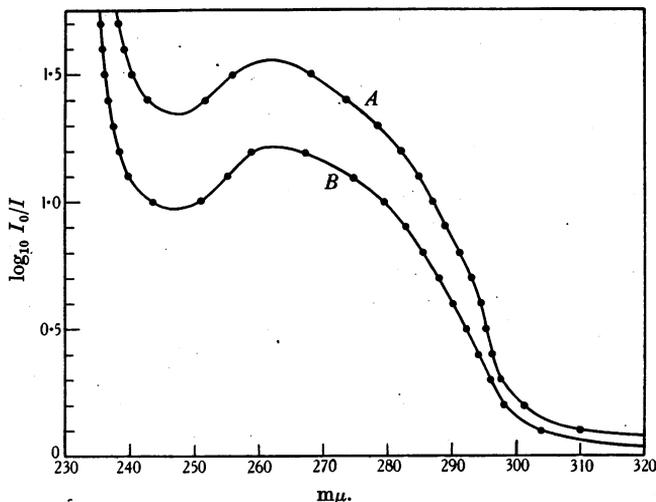


Fig. 1.

Changes similar to those with potato virus "X" occur when tobacco mosaic virus is inactivated with urea. On the other hand, absorption in the ultra-violet by tomato bushy stunt preparations is increased by denaturation with urea. The protein and nucleic acid are not separated when bushy stunt virus is treated with urea and the inactivated virus is insoluble in urea. Either of these differences between bushy stunt and the other two viruses may explain the difference in the behaviour of its absorption spectrum, for although in the absence of salts there is no actual precipitation of denatured protein, the fluid becomes more opalescent. When bushy stunt virus is inactivated by sodium dodecyl sulphate the protein and nucleic acid are separated and remain water-soluble, and there is an increase in the clarity of the solution. After this treatment the intensity of absorption of ultra-violet is increased, so that from this point of view the inactivation of bushy stunt by sodium dodecyl sulphate resembles the inactivation of tobacco mosaic virus and of potato virus "X" by urea.

#### DISCUSSION

In the experiments described in this paper urea has been regarded as the chief agent in causing denaturation. However, as denaturation requires the simultaneous control of several variables, this distinction is arbitrary. For example, as the rate of denaturation closely depends on the *pH*, it is as logical to regard urea as increasing the rate of alkaline inactivation as it is to regard alkali as increasing the rate of urea denaturation. Actually there is probably considerable interaction between the various factors. For example, our results show that at some *pH* values in the alkaline range, which do not themselves inactivate tobacco mosaic virus, concentrations of urea, which in neutral

solution do not inactivate, cause rapid inactivation. Also, one of the features of alkaline inactivation of tomato bushy stunt virus, loss of infectivity without loss of serological activity, occurs in less alkaline solutions in the presence of urea. Similarly, Drabkin [1939] compared the rates of denaturation of haemoglobin in dilute alkali, in 6*M* urea, and in a mixture of the two, and found that the mixture acted 60 times as rapidly as either component alone.

The restriction of the work reported to inactivation in the presence of urea is also largely arbitrary, for it is reasonable to assume that urethane, guanidine and related substances act in a similar manner and that there is a smooth transition to the somewhat remote denaturing agents such as pyridine, benzoates, salicylates, phenol and soaps. The effects of these substances on the plant viruses will be described in a later paper, but as little or nothing is known of the mechanism of their action it would be premature to attempt to classify them. There is one feature of inactivation in the presence of urea, however, that might justify the separation of urea from other denaturing agents and justify the view that urea is the chief agent in causing the denaturation rather than the other factors such as *pH*. This is the large increase in the rate of denaturation produced by cooling at temperatures below about 20°. This was found to be true for all the viruses we have tested, as well as for other proteins, but no such effect has been found for inactivation by alkali or other denaturing agents.

In our experiments only changes irreversible on dilution or dialysis and resulting in loss of specific virus activities have been investigated. The results show that for each virus there is a critical concentration of urea below which irreversible changes do not occur. This concentration varies with the different viruses, which, although they are all nucleoproteins, also differ in the manner in which they break down on denaturation. For example, the inactivation of potato virus "X" and tobacco mosaic virus is accompanied by the separation of the nucleic acid from the protein and the products are soluble in urea solution, whereas the inactivated bushy stunt and tobacco mosaic viruses are insoluble in urea and the precipitates still contain nucleic acid. In addition to these irreversible changes it is probable that urea can cause changes that are readily reversed and have no effect on virus activity. Frampton [1939] found that the addition of as little as 1*M* urea to solutions of tobacco mosaic virus greatly reduced the viscosity. As inactivation and disruption of the virus particles occur only slowly in much more concentrated urea solutions, it is probable that the immediate effect on viscosity is caused by the hydration of the particles and not by denaturation. We have found no evidence that the small particles produced by the disruption of the viruses possess virus activity, although the fact that purified virus is inactivated rather more slowly than impure virus may be evidence that the urea can dissociate the aggregates formed during purification as well as disrupt the actual virus particles.

#### SUMMARY

The literature on the effects of urea on proteins, tissues, bacteria and viruses is reviewed. The four viruses, tobacco mosaic, potato "X", tomato bushy stunt and tobacco necrosis, are irreversibly denatured by urea. The denaturation is accompanied by loss of infectivity and serological activity. For each virus there is a critical concentration of urea below which there is no irreversible effect on infectivity. This concentration is smallest for potato virus "X" and greatest for tomato bushy stunt virus. The rate of inactivation is greatly increased by the presence of alkali. The rate of inactivation is minimum at about 20° and is much increased by cooling to -10°. The inactivation of purified tobacco mosaic

virus by urea proceeds only slightly more slowly than that of virus in crude infective sap. The inactivation of tobacco mosaic virus and potato virus "X" is accompanied by separation of the nucleic acid and protein, but the inactivation of bushy stunt and tobacco necrosis viruses is not. Changes in the absorption spectra that accompany inactivation are described.

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