Denaturation of Proteins by Urea and Related Substances.*

By Sir F. GOWLAND HOPKINS, F.R.S.

UPON the facts enumerated the following simple method of determining the degree and rate of denaturation is based. A measured sample of the urea-protein mixture is diluted with ten times its bulk of water, and to secure complete precipitation of the denatured product a small quantity (say 1 gm. per 100 c.c.) of ammonium sulphate is added, and if precipitation is not immediate a small amount of acetic acid. When the precipitate has settled out it is filtered and washed, or better centrifuged and washed, until free from sulphate, when it will also be free from undenatured protein. The precipitate is then transferred to a tared basin, dried and weighed. The method is trustworthy and gives consistent results. If the protein left in solution after the precipitate has been removed be thrown out by saturation with ammonium sulphate, it will be found to be wholly resoluble in water; that is to say, it is undenatured albumin.

The rate of denaturation increases with increasing concentration of urea. With respect to the influence of protein concentration but few determinations have been made, but it may be said that within a fairly wide range of concentration the percentage denatured in a given time by a particular concentration of urea remains of the same order.

The only quantitative results which will be given here are those which bear upon the effect of temperature upon the process. These have special interest. In the experiments carried out to determine the rate of change, solutions containing about 5 per cent of protein have been employed and the urea added to 60 per cent of full saturation (of saturation, that is, at 15° C.; 0·6 gm. added per c.c.). With such proportions, while denaturation is rapid, there is for relatively long periods no spontaneous separation of the product, either as precipitate or gel.

The following results of two experiments are fully representative of many. After the addition of urea the solutions stood at the temperatures mentioned, and the amount of denatured protein determined at the intervals stated. It is given in percentage of the whole protein present. The concentration of albumin mentioned in the first column is that of the original solution, not that present after the increase of volume due to the addition of urea.

Experiment.	Temperature. (° C.)	Amount of Denatured Protein at Intervals after Addition of Urea (per cent of whole Protein present).		
		15 Min.	1 Hour.	3 Hours.
A. Albumin solution 5 per cent . Urea, 0·6 gm. per c.c. . . . *p*H before dilution, 5·9 . . .	0	79·0	86·2	92·8
	23	49·2	69·2	88·5
	37	..	61·0	85·1
B. Albumin solution, 4·14 per cent . Urea, 0·6 gm. per c.c. . . . *p*H before dilution, 6·0 . . .	0	78·3	85·7	91·0
	22	45·1	62·7	87·5
	37	..	58·1	82·8

It is seen that denaturation by urea is a rapid process at each temperature investigated. While, however, heat denaturation was shown in the classical experiments of Chick and Martin [5] to be a process with an exceptionally high temperature coefficient, the above figures present the simulacrum of a negative coefficient. At the concentrations employed nearly 80 per cent of the albumin is denatured in 15 min. at 0° C. and less than 50 per cent at 22° C. Data such as the above have been repeatedly obtained. There is no reversal of the process on any lines with increase of temperature. Denaturation in any case ultimately proceeds nearly to completion. Only such temperatures are, of course, to be considered as are well below those at which heat denaturation itself begins.

The provisional hypothesis which perhaps most readily covers such facts is that denaturation occurs in a protein-urea compound which, save in the presence of large concentrations of urea, is highly dissociated, and of which the dissociation increases with rise of temperature sufficiently to account for the observed diminution in the rate of denaturation. The increased dissociation must then be assumed to outweigh other temperature effects. Proof or disproof of such a view must depend upon a study of various equilibrium relations. This offers technical difficulties and results are not yet available.

Certain substances, which can be shown to denaturate egg albumin, differ from urea in that their solutions exert little or no dispersive action upon the denatured product. Thus, whatever the relative concentrations, when such substances are present in amounts sufficient to denaturate actively, the product separates rapidly as a precipitate. Urethane presents a case of this kind.

If a concentrated solution of urethane be gradually added to an albumin solution, a point is reached at which precipitation begins immediately. If then the mixture be allowed to stand, denaturation and separation of the product proceed rapidly, and a large proportion of the protein will be denatured in the course of a few minutes. Thus to a 5 per cent albumin solution (*p*H 4·8) urethane in strong solution was added at 20° C. Precipitation began when the mixture contained 18 per cent urethane, and in ten minutes the precipitate (a typically denatured product) was centrifuged, washed, and weighed. It amounted to 59 per cent of the original protein. Like urea, concentrated urethane acts very rapidly at 0° C. It denaturates, though more slowly, when a solution contains 10 per cent or less.

Thiourea also denaturates while displaying little dispersive power. To separate samples of 4 per cent albumin solution at *p*H 4·86 thiourea was added to saturation (9 per cent). On standing at 17° C. precipitates separated and were weighed at successive intervals. After 15 minutes about 12 per cent, after 3 hours 25·5 per cent, 24 hours 62 per cent, and after 48 hours 80 per cent of the protein proved to be denatured.

Although the effective concentrations of the denaturants under discussion is high, it is scarcely likely that a 'lyotrope' action plays any dominant part in producing their effects. The phenomena differ of course in fundamental aspects from that of 'salting out' by electrolytes.

The simultaneous presence of electrolytes in solution, though, on the whole, tending to diminish its velocity, does not seem to exercise an important influence upon denaturation by urea, at least in the case of molar concentrations which alone have been tried. It is possible that a study of their effects in higher concentrations might throw some light upon the process.

One other circumstance associated with the phenomena under discussion should be mentioned. Using the green line of the mercury vapour lamp, the optical

* Continued from p. 330.

rotation of protein solutions was found to increase (after making due correction for the resultant change of volume) more than threefold when saturated with urea. In three separate experiments the rotation was increased to 3·25, 3·45, and 3·30 times its original value in water. I refer to this here merely as a fact empirically established.

SERUM PROTEINS.

Fewer observations have been made upon these and less space must be given to their description.

Attention must first be directed to the circumstance that blood proteins, unlike egg albumin, do not when denatured by any form of treatment yield a nitroprusside reaction direct. If, however, the denatured products are afterwards exposed to reducing agents, they then give a colour reaction which is intense. The most convenient method of demonstration is to add a small quantity of potassium cyanide to the solution of denatured protein, or to soak a precipitate or gel in a 1-2 per cent solution of cyanide before, in each case, dissolving a little solid nitroprusside in the fluid. A justifiable assumption is that the precursor of the active thiol group is a disulphide grouping not present in the native protein but established on denaturation. The effects of cyanide, etc., are exactly similar to those observed in the case of cystine and its conversion into cysteine.[6]

If native serum (horse or sheep) or solutions of separated albumin and paraglobulins be saturated with urea and the mixture allowed to stand for a few minutes in the presence of a little potassium cyanide, the addition of nitroprusside solution (plus ammonia if the solution be not sufficiently alkaline) will then produce an intense colour reaction.

Serum or its separated constituents (the behaviour of euglobulins has not been studied) on evaporation with denaturants on the lines described for egg albumin, whether the *p*H be that of native serum or reduced, say, to 5, behave similarly to the latter. Substances which denaturate the one protein act upon the other : those which fail in one case fail in all.

When, however, their behaviour is observed in solution, it becomes clear that the blood proteins are definitely more resistant to denaturation on these lines than in ovalbumin. It is noteworthy that the difference is more marked when the influence of urea, itself, is in question than in the case of, say, urethane with its smaller dispersive power.

Serum in its native condition or when brought to a *p*H of the order of 5, if saturated with urea, sets in a few hours at room temperature, and somewhat more rapidly at 37° C., to a jelly, and so in strong solutions do serum albumin and globulin. Such jellies treated as described above give an intense nitroprusside reaction, but only gradually after more prolonged standing do they show the resistance to redispersion into sols, which is characteristic of ovalbumin gels. Nevertheless, diluted serum or solutions of serum albumin or paraglobulin containing, say, 4-5 per cent protein, when mixed with such concentrations of urea as will rapidly denaturate ovalbumin, yield even after several hours' standing no precipitate on dilution or dialysis, and show no signs when dialysed free from urea of having assumed the characters of the suspensoid condition. This remains true if the *p*H of the solutions before or after adding the urea is brought to near the isoelectrical point of the proteins when denaturated by heat, 5-5·4. Observations giving these results have been made with the proteins of sheep's blood and with crystallised albumin from horse serum. Only after remaining many days in contact with urea do the blood proteins show evidence of more complete denaturation. On the other hand, their solutions when mixed with high concentrations of urea give at once after treatment with cyanide an intense nitroprusside reaction. It would seem as though these proteins undergo with readiness the chemical changes which in all cases are associated with denaturation, but the colloid particles of the product are more resistant than those of egg albumin to the dehydration which characterises the change from the lyophil to the lyophobe condition.

Easily to be demonstrated, however, is the denaturation of blood proteins under the influence of urethane, especially, but not only, when they are brought near to their isoelectric point. The following figures are illustrative. Dialysed and filtered sheep serum was brought to *p*H 4·86 and urethane (0·3 gm. per c.c.) added. After 1 hour at 20° C. 22·5 per cent of the total protein was denaturated ; and after 3 hours 41·0 per cent. With thrice the concentration of urethane 43·5 per cent was denaturated in 1 hour. The process is clearly much slower than in the case of ovalbumin.

It is with intention that these notes have been confined to a simple description of observations which are themselves of a preliminary kind. Points of theoretical interest can scarcely fail to be noted, but at present they lack quantitative investigation. It has seemed worth while to give this indication that the phenomena in question are worthy of such investigation. Certain quantitative studies are being made in the Cambridge School of Biochemistry.

[5] *Jour. Physiol.*, **40**, 404 ; 1910.
[6] E. Walker, *Bioch. Jour.*, **19**, 1082 ; 1925.

Imperial Horticultural Conference.

AN Imperial Horticultural Conference, arranged by the Imperial Bureau of Fruit Production, met at the house of the Royal Society of Arts on Aug. 5-7.

The papers presented to the Conference were grouped according to the aspect of horticultural work discussed. In the group dealing with field experimentation, Mr. T. N. Hoblyn, of East Malling, stated that the failure of earlier research on fruit trees was due to (1) the inherent variability in the trees themselves, (2) variation due to outside causes. These causes of error can now be eliminated by the adaptation of statistical method to known material raised clonally. Prof. E. E. Cheesman, Imperial College of Tropical Agriculture, Trinidad, stated that the same inherent variability is markedly noticeable in tropical crops, which are largely cross fertilised and heterozygous. Here, too, in dicotyledons, clonal propagation seems to offer a solution ; cacao is at present under investigation at the Imperial College at Trinidad. In the subsequent discussion, emphasis was laid on the desirability of close contact between the statistician and the horticultural worker, and on the importance of the close observation of individual trees in horticultural experiments.

Dealing with the application of the pure sciences to horticultural problems, Prof. B. T. P. Barker, Long Ashton Research Station, Bristol, remarked that chemistry can help the cider industry, particularly by determining the constituents of the apple ; apples other than pure cider varieties can be used to supplement these. Investigations are in progress on the substitution of centrifuging for filtering. Prof. V. H. Blackman, Imperial College of Science and Technology, London, said that the physiological study