STUDIES OF THE REACTION BETWEEN PROTEINS AND REDUCING SUGARS IN THE "DRY" STATE

I. THE EFFECT OF ACTIVITY OF WATER, OF p_H AND OF TEMPERATURE ON THE PRIMARY REACTION BETWEEN CASEIN AND GLUCOSE

by

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While it is now believed that products of a reaction between the free amino groups of amino acids or proteins and reducing sugars may contribute to the desirable colour, aroma and flavour of certain processed foods, they are apparently concerned also in the deterioration of a number of other food products and the reaction has obvious possibilities of interest in non-food systems containing protein or protein fragments and carbohydrate.

In recent work on the deterioration of skim milk powder HENRY, KON, LEA, AND WHITE[1] obtained clear indications of a reaction between the free amino groups of the milk protein and lactose leading to undesirable physical and chemical changes in the powder and to a reduced availability to young rats of the lysine present. The use of a simpler system than milk powder, however, was essential for further elucidation of the changes involved and a general investigation has therefore been undertaken of the interaction between proteins and reducing sugars. Casein was chosen as starting material for the first experiments, despite its known lack of homogeneity, because it accounts for over 80% of the protein of milk, and because the quantities of material required precluded the use of crystalline β-lactoglobulin. This latter protein is present as only a very minor constituent of milk protein, and is now itself believed to be heterogeneous.

Practically nothing appears to have been published on the reaction between protein and sugar at low activities of water, and very little on the reaction in solution. SHIGA[2], in a single experiment with egg albumin and glucose in solution (100 moles glucose per free amino group) observed only a comparatively slight reaction which increased with increasing p_H over the range p_H 7–9. Several earlier workers had also obtained evidence by various methods of a slight combination between glucose or fructose and proteins in aqueous solution at p_H values more alkaline than 7. PRZYLECKI AND CICHOCKA[3] investigated the formation of "covalence-like symplexes" from carbohydrates and proteins in aqueous solution by allowing protein solutions of 5–7% concentration to react with saturated or half saturated sugar solutions at 5–12° C and at a p_H initially of 7–9 and finally of 9–10 for 2–4 days. A marked proportionality was reported between the

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lysine content of the protein used and the amount of sugar found in the product after precipitation with alcohol. The combination however appeared to be unstable and was split at physiological p_Hs.

A considerable amount of information is available concerning the reaction between amino acids or simple peptides and sugars in solution, but is often conflicting. Evidence for combination has been obtained by cryoscopic, optical rotation and electrometric titration methods, as well as by the loss of VAN SLYKE nitrogen and by the increase of reducing power towards methylene blue or 2:6 dichlorophenolindophenol. The reaction is slow and does not approach completion even after many hours at laboratory temperature. At high concentrations and temperatures extensive secondary reactions occur leading to the production of complex, highly coloured substances of high molecular weight ("melanoidins"), and eventually to charring and the copious evolution of gas.

Evidence as to the effect of p_H on the rate of reaction has been conflicting. Some of the earlier data show a marked optimum in the region of p_H 7–8[4, 5], while others indicate a continued increase in the rate of reaction with increasing alkalinity[6, 7]. SHIGA[2] concluded that the amount of combination of amino acid with glucose increases with the p_H value, while that of di- or tripeptides with glucose has an optimum at p_H 7 or 8. FRANKEL AND KATCHALSKY[8] found that the percentage combination at equilibrium of glucose with glycine ranged from zero at p_H 6 to over 80% at p_H 10 and above, while for leucylglycine the corresponding p_H values were 7 and 11.5, no p_H optimum being observed in either case.

Free amino groups are not the only reactive centres in the protein molecule which might combine with aldehyde groups. With cysteine in aqueous solution, for example, the SH as well as the NH_2 group has been shown to react with glucose to give a fairly stable thiazole ring compound which has been isolated[9, 10].

METHODS

Sodium caseinate

Casein was prepared from fresh, unheated cow's milk, after centrifugal separation of the cream, by the method of COHN AND HENDRY[11], which avoids exposure to organic solvents or to alkaline conditions. After precipitation at p_H 4.6 and washing, the isoelectric casein was dispersed again by the slow addition, with stirring, of sodium hydroxide to p_H 6.3, and the "solution" carefully dried in the frozen state under high vacuum. The product, after removal from the drying trays was held overnight in an atmosphere at 60% R.H., broken down and mixed by very brief agitation with the stainless steel blades of a WARING blender, and stored in sealed containers at −20° C until required. The sodium caseinate thus prepared consisted of light white glistening flakes which were readily dispersible in water.

Casein-glucose mixtures

For the preparation of 'dry' casein-sugar reaction mixtures part of the stock sodium caseinate was dispersed in water to a 2% solution, glucose was added in quantity exactly equivalent to the determined free amino N content of the protein (*ca* 11% by weight of the anhydrous protein) and the liquid, after cooling to 0° C, was shock frozen by rapid evaporation in the freeze-drier. In some cases the p_H of the liquid was adjusted to some desired figure by the addition of HCl or NaOH before drying. After freeze-drying the samples were held at 0° C in an atmosphere at 56% R.H. (corresponding to approximately 60% R.H. at 20° C) for 2 days to reduce moisture changes during subsequent handling, and mixed in the WARING blender before use. Owing to the relatively enormous surface area exposed by these products they could readily be equilibrated with any desired atmosphere.

Equilibration to known water-vapour pressures

Since it was considered likely that the influence of water on chemical reactivity within the system would be connected more closely with the activity of water as indicated by vapour pressure measurements than with total water content as determined by some arbitrarily chosen heating or drying method, storage conditions have been defined, wherever possible, in terms of the vapour pressure of

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the material expressed as percentage relative humidity. Since, moreover, the rate of transference of moisture from environment to sample or vice-versa has a much smaller temperature coefficient than have the chemical reactions under investigation the sample was usually equilibrated over dilute sulphuric acid solution to the required moisture content at a low temperature prior to storage at a higher temperature.

The pressure of water-vapour over solutions of sulphuric acid of known concentration changes regularly with the temperature, and the magnitude of the effect is comparatively small. Proteins, however, show so marked a change in capacity for 'binding' water with change in temperature that some preliminary investigation with the experimental material to be used was necessary.

The water-relations of the casein-glucose system

Investigation of the water relations of the casein-glucose system was complicated by the necessity for equilibrating the samples sufficiently rapidly to avoid changes resulting from commencement of the amino-aldehyde and its secondary reactions. This meant the use of small weights and large surface areas, which militated against the attainment of a high degree of accuracy. The data given in Fig. 1 were obtained by bringing the weighed samples of casein-glucose into equilibrium first with an atmosphere of 85 % R.H. and subsequently with the required relative humidity at 10° C. During the 2 or 3 days required for this process chemical change was negligible. The samples were then transferred to atmospheres at 37° C so chosen that only a very small further quantity of water would be given up, and weighed *in situ* at intervals up to 48 hours. From the curves obtained by plotting change in weight against time a correction for any loss of volatile matter due to commencement of the chemical reaction could be made. In general, the correction was zero or very small for the driest samples, but was quite appreciable in the region of maximum reaction velocity (*ca* 70 % R.H.). The moisture contents of the various samples were then obtained by relating them to the original material, the water content of which was found by drying in vacuo over anhydrone (magnesium perchlorate) at 0° C for 3 weeks and then at 37° C to constant weight.

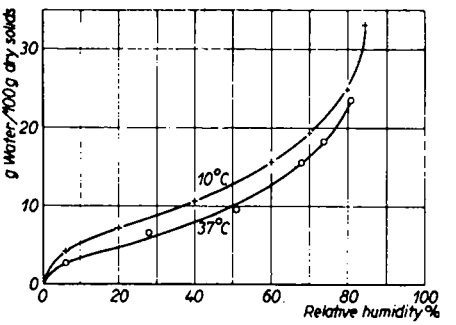

Fig. 1. The relation between water content and equilibrium relative humidity for the casein-glucose system at pH 6.3

At temperatures above 37° C the rate of chemical change was so great that adsorption data could not be obtained directly. The isotherms required at 55, 70 and 90° C were therefore derived with sufficient accuracy from those at 10 and 37° C by the extrapolation of isosteres on a logarithmic plot[12]. With the aid of the appropriate isotherms samples of casein-glucose could be adjusted at 10° C to water contents which would be in equilibrium with a wide range of atmospheres at higher temperatures, and the initial stages of the reactions would not therefore be complicated by unnecessary gain or loss of water.

Storage

a) *Constant relative humidity.* Many of the experiments were carried out in a room with rapid air circulation, thermostatically controlled at 37 ± 0.1° C. Six or eight 200 mg samples in thin-walled glass tubes, after equilibration at 10° C were stoppered, heated rapidly to 37° C, opened and transferred to airtight jars containing sulphuric acid producing the required relative humidity. A similar technique was employed for storage at 28.5, 20, 10 and 0° C.

The experiments at 55° C/70 % R.H. and 70° C/70 % R.H. were carried out in an airtight jar partly filled with sodium nitrate solution of suitable strength, and maintained at reaction temperature by total immersion in a water thermostat. The samples, after adjustment to the correct moisture content at 10° C were compressed against the flat bottoms of the tubes to improve thermal contact with the glass, and rapidly heated by partially immersing the tubes in the sodium nitrate solution. A thin film of Silicone grease on the outsides of the tubes prevented 'creeping' of the salt solution into the contents.

b) *Constant moisture content.* Samples of 100 mg each, with moisture contents adjusted to 6.0, 10.3 and 13.9 %, were heated in closed containers in which the free space was so small that it could be saturated at reaction temperature by the evaporation of less than 2 % of the water present in the material. At 37° C the material was packed into glass specimen tubes of volume approximately 2 ml, closed with rubber stoppers and sealed with wax; at 70 and 90° C it was compressed into discs approximately 2 cm in diameter and 0.5 mm thick between two sheets of pure tin foil which were then sealed by a triple fold along the open edges. This latter type of package was used because it not only had a small internal volume but could also be heated rapidly between two heavy copper blocks (*ca* 3 kg each) contained in an electric oven. Both blocks were drilled for thermometers and the upper carried an insulated handle to facilitate rapid movement. After heating for the desired period the

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foil-wrapped sample was removed, chilled between cold metal blocks and weighed (for estimation of loss of moisture) prior to chemical examination. This technique is an adaptation of that employed by WRIGHT[13] in an investigation of the effect of heat on the solubility of milk powder.

Estimation of free amino groups

The determination of free amino-N was carried out by the manometric method of VAN SLYKE according to the procedure previously described[14], employing a reaction time of 30 minutes at 20° C and a correction of +2 mg amino-N/g total N to allow for completion of the reaction. The dry sample was usually dispersed by 'wetting' with 1 ml of glacial acetic acid in the graduated cup of the apparatus, followed immediately by dilution with water and washing into the reaction chamber. There was no evidence that this procedure caused any liberation of combined amino groups. Alternatively, the sample was dispersed in water by standing for three hours in a 5 ml cup fitted with a tap, through which the viscous solution could be drawn into the apparatus. This method was used, after a preliminary shredding with scissors, for the thin discs of compressed material produced by the copper block technique.

Measurement of colour

The stored samples were packed in a standardized manner into small porcelain dishes and examined in the LOVIBOND-SCHOFIELD Tintometer. Illumination was by C.I.E. Standard Illuminant B, consisting of gas-filled metal filament lamps operating at a colour temperature of 2848° K, used in combination with the specified colour filter solutions. Little use of the obturator vane was necessary over the limited range of brightness encountered in the experiments and, for simplicity, colours have been recorded as the sum of the yellow and red units used.

RESULTS

CHANGES IN FREE AMINO-N

Effect of activity of water on reaction velocity

The results summarized in Figs 2A and 2B were obtained in two experiments covering respectively 0–64 and 0–2 days at 37° C. In the first, casein-glucose weighed at 60% R.H. was adjusted to the required moisture contents and relative humidities without any pre-treatment. Some of the samples therefore reached equilibrium by hydration, and some by dehydration. It is known that a number of proteins display a slight hysteresis between 15 and 65%[15], material equilibrated by dehydration retaining appreciably more moisture than similar material equilibrated to the same relative humidity by hydration. In the second experiment, therefore, the casein-glucose was roughly equilibrated at 13–15% R.H. before adjustment to the required value, so that all the samples attained equilibrium by absorption of water; the data for 1 and 2 days in Fig. 2B appertain to this experiment. No major discrepancy between the rates of reaction in the two series of samples is apparent.

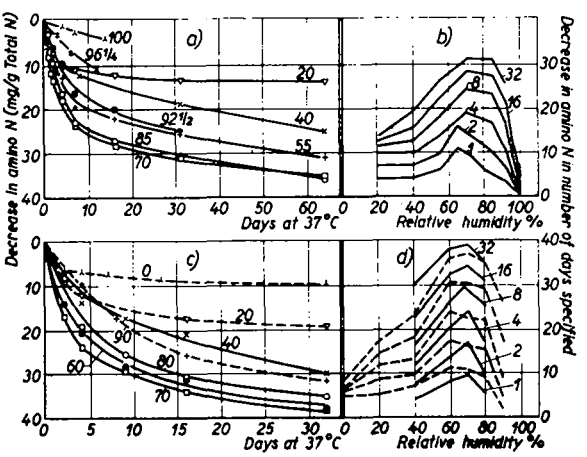

Fig. 2. The relation between activity of water and loss of free amino-N in the casein-glucose system at 37° C and pH 6.3. A and B were obtained without pre-adjustment of water content, C and D by dehydration (continuous line) or hydration (broken line).

While this work was in progress, the data of MELLON, KORN, AND HOOVER[16] appeared, according to which the hysteresis water of isoelectric casein, which can amount to as much as 2% at 51% R.H., is still present to the extent of approximately

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1.2% at 6% R.H. and requires virtually complete drying for its removal. A double range of samples was therefore prepared from a fresh preparation of sodium caseinate for a third experiment. For the dehydration series, the weighed samples of casein-glucose were equilibrated at 10° C and 85% R.H. before adjustment to the correct moisture contents and transference to 37° C for storage at the required relative humidities. For the hydration series, the casein-glucose was dried as intensively as was practicable without inducing chemical change (anhydrone in vacuo for several weeks at 0° C followed by 4 days at 20° C) before adjustment of the moisture contents at 10° C and storage at 37° C.

Changes in free amino-N during storage of the dehydration and of some of the hydration materials are shown in Fig. 2C; the remaining hydration curves are omitted for the sake of clarity. While the behaviour of dehydration and hydration samples on storage was not identical (Fig. 2D), the differences were considered to be of doubtful significance and it was concluded that the rate of disappearance of the protein amino groups was mainly determined by the equilibrium relative humidity of the system and was not seriously influenced by the route by which equilibrium had been attained. Measurement of the weight of the samples at intervals during storage failed to indicate any considerable difference between the water contents of the corresponding hydration and dehydration materials. It must be borne in mind, however, that any conversion of glucose from the hygroscopic supercooled "glass" to the comparatively non-hygroscopic crystalline form, which might occur as a result of exposure to a moist atmosphere, would tend to counterbalance the effect of hysteresis in the protein.

To obtain 0% R.H. the casein-glucose was dried over anhydrone at 0° C for several weeks before storage over anhydrone at 37° C. A sample dried for a shorter period and stored over 'concentrated' sulphuric acid, which also should provide an atmosphere of virtually 0% R.H., showed an appreciably higher rate of reaction, thereby emphasizing the difficulty of removing chemically active water from the nearly dry protein. Toluene and chloroform were present in the atmospheres of the vessels containing the 100, 96¼, 92½ and 90% R.H. samples, in order to retard microbial attack. In separate runs at 85% R.H. with and without the antiseptic no difference in reaction rate attributable to its presence could be detected. The '100%' R.H. sample, which was stored over water, was still absorbing water at the end of the experiment. The low reaction rate observed for this material (Figs 2A and 2B) must, therefore, have been too high. A sample stored in concentrated aqueous solution in the presence of toluene showed a very slow and approximately linear *increase* in free amino N, presumably as the result of hydrolysis.

The results at 92½ % R.H. and below disclose a relatively rapid initial drop in amino-N, varying greatly in rate and extent with relative humidity, and falling away after 5–15 days to a slow drift downwards. The data at 96¼ and 100% could not be completed because of the appearance of signs of microbial attack, but showed a much slower initial rate of reaction. Figs 2B and 2D, in which the reduction in free amino N after various periods of storage has been plotted against the relative humidity of the atmosphere in equilibrium with the sample, show that the initial rate of combination of the free amino groups of casein with glucose displays a comparatively sharp maximum in the region of 65 or 70% R.H., and falls away at higher and lower humidities. At low moisture contents the reaction slows down or stops after only a small proportion of the free amino groups have reacted.

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Effect of p_H

Portions of the stock sodium caseinate (p_H 6.3), after dispersal in water and the addition of one equivalent of glucose, were adjusted to p_H values in the regions of 2, 3, 4.6, 7, 8, 9 and 10, shock frozen, freeze dried, equilibrated at 0 or 10° C, and stored at 37° C and 55 or 70% R.H. Sodium hydroxide solutions were used for controlling the vapour pressures of the more alkaline samples. Control samples of protein alone at p_H 3, 9 and 10, without glucose, remained unchanged in free amino group content during storage for over 2 months at 37° C and 70% R.H. A decided tendency was noticed, however, for the amino-N content of the most acid samples, before storage, to be higher than the normal initial value of 53–55, and the rate of combination of protein and sugar at p_H 2 could not be determined with any accuracy, since at this acidity the apparent free amino content of a casein-glucose mixture (as well as of casein alone) *increased* during storage at 37° C and 55% R.H., possibly as a result of the conversion of amide groups to ammonia which is known to interfere with the VAN SLYKE deter-

TABLE I

THE EFFECT OF p_H ON THE RATE OF LOSS OF FREE AMINO-N IN CASEIN-GLUCOSE AT 37° C AND 55 OR 70% R.H.

p_H	Initial rate of loss of amino-N*	
	55% R.H.	70% R.H.
3.0	4	7
4.6	—	14
6.3	35	44
7.0	41	68
8.0	55	71
9.0	53	—
10.0	63	—

* Reciprocal of the number of days required for reaction of 20% of the free amino-N × 100

mination[17]. Correction for the 'blank' determination without glucose indicated a rate of reaction at p_H 2 even slower than the already slow rate obtaining at p_H 3 and 55% R.H. Measurement of the rate of reaction between casein and glucose at p_H 4.6 and 70% R.H., was also unsatisfactory owing to the impossibility of making an intimate homogeneous mixture as casein is virtually insoluble at this p_H, and the value obtained may be low.

Table I shows that the initial reaction between casein and glucose is comparatively slow under acid conditions, and increases with increasing p_H at least as far as p_H 8. The course of the reaction beyond this point was difficult to ascertain by the technique employed. Storage at 55% R.H. for example showed, in one experiment, a much slower rate of increase (Table I), and in another even a slight falling off in reaction rate between p_H 8 and 10. The freshly prepared materials at p_H 9 and 10, however, possessed appreciably lower initial amino-N values than did the samples at p_H 6.3, 7 and 8, and if the experimental data were corrected on the assumption that the difference in initial values (2½ and 4 units in the case of the p_H 10 samples) represented reaction of very labile groups during preparation then a comparatively smooth extension of the p_H 3–8 curve was obtained.

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At 70% R.H. much larger decreases in reaction rate above p_H 8 were observed and when combination became very slow after about 30 days at 37° C the amino-N content of the p_H 9 and p_H 10 samples had only fallen by some 26 and 19 units respectively, in place of the 34 units observed at p_H 6.3, 7 and 8. Considerable losses of free amino-N observed in samples at p_H 9 and 10 during preparation and equilibration prior to storage were again suggestive of a very rapid reaction at alkaline p_H of some of the free amino groups present.

It seems reasonable to conclude therefore, on the evidence available, that the rate of combination of the free amino groups of casein with glucose probably continues to increase with increasing alkalinity beyond p_H 8, although the methods used were unable to establish the point with certainty.

Effect of temperature

Casein-glucose, pre-dried to 13% R.H. for four days at 0° C before adjustment at 10° C to the required moisture content, was held in equilibrium with an atmosphere of 70% R.H. at temperatures of 0, 10, 20, 28.5, 37 and 55° C, as described above, and examined at intervals for free amino-N content and for colour. Above 55° C the reaction became so rapid that appreciable errors could be introduced during the brief time required for the samples to attain reaction temperature and to adsorb or desorb any small quantities of water necessitated by slight errors in the isotherms. A further experiment was, however, performed at 70° C to obtain a figure of less accuracy.

When the results were plotted it was found that all the curves of amino loss against time were of the same general shape and tended to the same final value. The rates could therefore be compared quite adequately by considering only the initial reaction rate determined by drawing the tangent at zero time to the smoothed curve. A number of readings were taken at short reaction times to ensure the accuracy of the initial portion of the curve. The values for the initial rate, expressed as % loss of amino-N per hour, were 0° C–0.0015; 10° C–0.0067; 20° C–0.034; 28.5° C–0.21; 37° C–0.71; 55° C–9.3; 70° C–58.

A second series of samples was heated at constant moisture content, partly for comparison with the constant relative humidity results and partly because this system was the only practicable one at the higher reaction temperatures. The casein-glucose was first dried over magnesium perchlorate at 10° C, equilibrated at 10° C to three different moisture contents of 6.0, 10.3 and 13.9% and then heated at 37° C in glass tubes or at 70 or 90° C between copper blocks as previously described. The accuracy of the method at high temperatures was limited by the fact that the tinfoil packets allowed small losses of moisture over long periods, and by unavoidable errors of the order of ± 5 seconds in the time and ± 0.2° C in the temperature of heating. Each reading, however, was taken in triplicate, and the general agreement was found to be good. Smooth curves were plotted and the initial rates determined as before. Casein heated alone at 90° C for one hour under the conditions of the experiment showed no significant loss of amino-N.

The results of the two experiments are summarized in the ARRHENIUS plots of Figs 3A and 3B, and the following conclusions can be drawn.

a) The data at constant relative humidity show a good straight line relationship, with a temperature coefficient of 5.4 between 15 and 25° C, or 3.6 between 60 and 70° C. A value of 29000 cal for the apparent energy of activation can be deduced from the slope of the graph.

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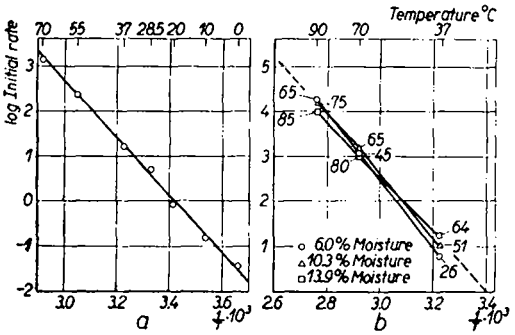

Fig. 3. The relation between temperature and the initial rate of loss of free amino-N in casein glucose (A) at a constant relative humidity of 70% and (B) at constant water contents of 6.0, 10.3 and 13.9%. (Rate is measured in % loss of free amino-N per day. Figures on graph (B) are the approximate relative humidities at the specified temperatures).

b) Whereas at 37° C the sample containing 13.9% of moisture reacted fastest, and that containing 6.0% was slowest, the opposite applied at 90° C. At 70° C the 10.3% sample was faster than the other two. These apparent discrepancies disappear if the actual relative humidity at the temperature of reaction is considered. The figures obtained from the isotherms (derived by extrapolation from data at 10 and 37° C) are shown against the individual points in Fig. 3B, and although these values can only be approximate they suffice to show that at high as well as at low temperatures the maximum reaction rate occurs in the region of 65% R.H. and falls away at humidities above and below this value.

c) The reaction rates at 70 and 90° C are sufficiently consistent with those observed at lower temperatures to indicate that the rate increases uniformly with temperature from 0 to 90° C.

CHANGES IN COLOUR

The effects of the activity of water at a constant p_H of 6.3, and of the p_H of the system at activities of water corresponding to 70% R.H., on the development of a brown discoloration in casein-glucose at 37° C are shown in summarized form in Table II. The development of colour in all cases was preceded by a lag period which became progressively shorter with increasing R.H. or p_H. This lag period was followed by an initial

TABLE II

THE EFFECT OF ACTIVITY OF WATER AND OF p_H ON THE DEVELOPMENT OF COLOUR IN CASEIN-GLUCOSE

% R.H. (at p_H 6.3)	Lovibond Y + R units after days at 37° C					
	2	4	8	16	32	64
20	—	—	—	—	—	0.2
40	—	—	—	0.0	0.1	0.6
55	—	—	—	0.1	0.8	1.5
70	—	—	0.0	0.8	1.9	2.7
85	—	0.1	0.8	1.9	3.7	5.0
92½	—	0.1	0.8	1.9	3.3	—
96¼	—	0.1	0.8	2.1	—	—
p_H (at 70% R.H.)						
3.0	—	—	0.1	0.2	0.5	1.1
4.6	—	—	0.2	0.4	1.0	1.9
6.3	—	—	0.1	0.9	1.8	2.6
7.0	—	—	0.3	1.1	2.0	2.9
8.0	—	0.0	0.7	1.6	2.4	3.4
9.0	0.1	0.4	1.2	2.2	3.0	3.8
10.0	0.2	0.8	1.7	2.6	3.4	4.4

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rate of development of colour which was more rapid the higher the R.H. or p_H but which tended to fall away progressively with time, except under very acid conditions (p_H 3 and 4.6) when the rate of increase of colour appeared to be almost linear. The development of colour by protein in the absence of sugar under the conditions of the storage experiments was negligible.

The colour data show no sign of the comparatively sharp maximum in initial reaction velocity observed in the region of 65 or 70% R.H. for the disappearance of free amino-N (Fig. 2). Instead, colour development was approximately twice as rapid at 85, 92½ and 96¼%, the highest humidities at which it could be measured, as at 70% R.H. The measurement of colour became increasingly less satisfactory at humidities higher than 85%, owing to changes in physical structure of the material leading to shrinking and 'stickiness'.

Table II shows that at 37° C and 70% R.H. the rate of discoloration increased continually with increasing p_H from 3 to 10. At 55% R.H. results were generally similar, the rates at p_H 3, 6.3 and 10 being of the order of 45, 65 and 80% respectively of the corresponding rates at 70% R.H.

The effect of temperature cannot be reported in detail, the different physical states of the samples after the various temperature treatments rendering accurate colour comparison difficult. It was evident, however, that the progressive increase in rate of colour development with increasing water content occurred at all temperatures, the samples containing 13.9% water discolouring more rapidly than those with 10.3 and 6.0% even at 90° C, where the 6% sample showed the most rapid loss of amino-N. At a constant relative humidity of 70% the temperature coefficient was of the same order as that for the disappearance of the amino-N, and the initial lag period persisted at all temperatures. There was some indication, however, that the temperature coefficient was slightly higher than for the amino reaction, and that the lag period was relatively slightly less at the higher temperatures.

CHANGES IN SOLUBILITY

In the case of the reaction between reducing sugars and dialysed milk proteins at 37° C[18] it was observed that after a brief initial lag period the protein rapidly became insoluble. The casein-glucose complex on the other hand has a definite affinity for water even when deterioration is marked and considerable colour has developed. Samples with more than half their free amino-N remaining were found to be completely soluble at a concentration of 5%, and even after long storage at 37° C the material could still be reconstituted to a viscous gel. After very long storage at high temperatures however (*e.g.*, 80 minutes at 90° C) water imbibition was slow even in the presence of acid, and for practical purposes the material could be regarded as being insoluble. The relationship of the development of insolubility to the amino-reducing sugar reaction is being further investigated.

DISCUSSION

In all of the experiments it has been considered advisable to base conclusions mainly on the earlier stages of the reaction, owing to the probability of partial destruction of the glucose by side or consecutive reactions on continued storage, and to the

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possibility of interaction between protein amino groups and sugar degradation products.

The most striking feature of the reaction between casein and glucose appears to be the nature of its dependence on the activity of water in the system, whereby a maximum rate is observed at a moisture content corresponding to an atmospheric humidity in the region of 65 or 70%, while the change is very slow at very low or very high humidities. Since a mild dehydration treatment only could be applied to the casein-glucose mixture it seems quite likely that the small initial reaction observed at 0% R.H. (Fig. 2) was due to water still retained by the protein and that the reaction rate in the absence of moisture would be zero. As the water content increased the rate of the initial reaction and the extent of the change before the reaction slowed down or stopped both increased. After passing the optimum R.H. the initial rate of reaction fell away again quite sharply, but the level of amino-N at which the reaction slowed down or stopped could not be ascertained in this case because of the onset of microbial decomposition.

The relative humidity at which the reaction rate reaches a maximum corresponds with the end of the approximately linear portion of the adsorption isotherm, above which the curve swings steeply upwards (Fig. 1). BULL[15] has suggested, on the basis of the general multilayer adsorption theory of BRUNAUER, EMMETT, AND TELLER[19], that this point on the isotherm of proteins represents the completion of a double layer of water molecules between the protein planes, and probably also the point at which the exposed polar groups of the protein have become saturated with water. Under these conditions the force of attraction between the protein molecules will be greatly decreased and their capacity for movement and re-arrangement increased*. As the water vapour pressure is further increased the protein will tend to go into solution. MELLON, KORN, AND HOOVER[16, 20] have recently attempted to carry the matter further by determining the proportion of the bound water which is associated with the free amino groups of isoelectric casein. They conclude that at very low water vapour pressures (0–6% R.H.) one molecule of water is probably held between two amino groups. As the water vapour pressure increases the quantity of water held by the amino groups increases linearly to reach saturation of the hydrogen bonding capacity at about 2½ molecules, per amino group at 60–70% R.H. Above 70%, and particularly above 80% R.H. there is a rapid increase in the water adsorbed on the amino groups, probably by condensation of water on the water molecules already bound. A fraction varying from one quarter to one third of the total water held by the protein at various humidities was considered to be associated with the amino groups, but this included none of the water responsible for the phenomenon of hysteresis.

On the basis of these data is it not difficult to account for an increasing rate and extent of the reaction between casein amino groups and glucose with increasing R.H. up to 65 or 70%, but less easy to understand the marked falling off in reaction rate as

* In this respect the work of BARKER[21] on the effect of relative humidity on the temperature of denaturation of egg albumin, and on the effect of denaturation on the water relations of albumin is of interest. BARKER equilibrated his samples of egg albumin to a wide range of relative humidities at room temperature, heated them in sealed tubes at 60 to 160° C for 10 or 60 minutes and deduced a straight line relationship between the temperature required for denaturation and the relative humidity. The samples were therefore heated at constant moisture content and, if allowance be made for the large effect of the high temperatures used on the equilibrium R.H. of the system, it becomes clear that denaturation only occurred above 65–70% R.H. Recalculation of the data in detail has not, however, been attempted, since no account appears to have been taken in the experiments of loss of water into the free space above the samples, which may have influenced the results seriously at the higher temperatures.

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the humidity continues to increase beyond this point. It may be that a simple dilution effect is coming into play after sufficient water has been added to bring all the glucose into solution, or that an increasing thickness of aqueous film is tending to keep apart amino and potential aldehyde groups which, while both hydrophylic, have no great affinity for one another as evidenced by their slow and incomplete reaction in aqueous solution. Separate experiments which showed that the rate of reaction at 70% R.H. can be considerably increased by increasing the concentration of glucose above the level employed in these experiments would seem to militate against the simple solubility theory.

The conflicting nature of the conclusions drawn from previous investigations of the effect of p_H on the reactions between sugars and amino acids or peptides in aqueous solution has already been commented upon (page 314), and the problem is not simplified by the substitution of protein for amino acid and of the "dry" state for aqueous solution. The term p_H itself is of very doubtful significance in the presence of so little water, and it has been used in the present work only to indicate the reaction of the aqueous dispersion, from which the solid reaction mixture was prepared by freeze drying. Inevitably, this value will tend to fall as the strongly basic amino groups react, and it may be depressed further by the formation of acidic degradation products from the sugar. With protein, however, these changes are comparatively small below p_H 8, and are unlikely to influence the reaction perceptibly in its earlier stages; at p_H 9 and 10 a more marked fall in p_H commences during drying and continues during storage. The effect of p_H on the reaction between "dry" casein and glucose differs from that found by FRANKEL AND KATCHALSKY[8] for amino acids or peptides and glucose in aqueous solution in that it proceeds at a very appreciable rate even under quite strongly acid conditions, but is similar in that it increases with increasing p_H at least as far as p_H 8, and probably up to p_H 10.

The temperature coefficient of 5.4 between 15° and 25° C. for the casein-glucose reaction is somewhat lower than the value of "at least 6" deduced from the earlier experiments on the deterioration of stored milk powder[1], but this latter figure included a contribution from a change in relative humidity due to the crystallization of lactose, a process which possessed a higher temperature coefficient even than the amino-sugar reaction.

The production of a brown colour from the protein-glucose mixture only when loss of free amino groups occurs, and under conditions where protein and sugar alone are stable, coupled with the existence of a distinct "lag" in the appearance of colour after the fall of amino-N has commenced, support the view that discoloration results from secondary changes in a first formed, colourless protein-glucose complex. The maintenance of a high rate of colour production as the relative humidity is increased above 70%, while the primary reaction between the amino groups and glucose is slowing down, suggests further that increasing concentrations of water considerably beyond 70% R.H. have an accelerating effect on the secondary reactions leading to discoloration. The mechanism of the production of colour will, however, be considered further in work on the preparation and properties of the casein-glucose complex now in progress.

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SUMMARY

1. The reaction between casein and glucose (one molecule per free amino group of the casein) in the "dry" state has been studied by determination of free amino-N and measurement of changes in colour.

2. The rate of loss of amino-N has been found to depend on the following factors:

a) It is powerfully influenced by the activity of water in the system, showing a maximum at water contents corresponding approximately to 65 or 70% relative humidity, and falling away to low values at very high and very low water contents. This relationship has been found to apply at 37, 70 and 90° C.

b) It increases with p_H from low values at p_H 3 up to p_H 8 at least, and probably up to p_H 10.

c) It shows a progressive increase with temperature from 0 to 90° C and, when the relative humidity is kept constant at 70%, conforms to the ARRHENIUS equation with a $Q_{10}^{15-25°\,C}$ of 5.4.

3. The development of colour increases with increase of water content, p_H and temperature.

4. Possible mechanisms relating the effects of water to the multilayer adsorption theory of BRUNAUER, EMMETT, AND TELLER are discussed.

RÉSUMÉ

1. Nous avons examiné la réaction entre la caséine et la glucose (1 molécule par groupe aminé libre de la caséine) en état "sec" par la détermination de l'azote aminé libre et par le mesurage des changements de couleur.

2. Nous avons observé que la vitesse des pertes de l'azote aminé dépend des facteurs suivants:

a) Elle est puissamment influencée par l'activité de l'eau dans le système. Elle est au maximum quand le contenu aqueux correspond à une humidité relative du 65 ou 70%; elle est petite quand le contenu aqueux est très élevé et quand il est très réduit. Cette relation a été observée à des températures de 37, 70, et 90° C.

b) Elle augmente avec le p_H, de petites valeurs à p_H 3, jusqu'au moins à p_H 8, sinon à p_H 10.

c) Elle augmente progressivement avec la température de 0 à 90° C, et, si l'humidité relative reste fixée à 70%, elle est conforme à l'équation d'ARRHENIUS avec $Q_{10}^{15-25°\,C}$ de 5.4.

3. La coloration augmente avec l'augmentation du contenu aqueux, du p_H et de la température.

4. Nous examinons la question des mécanismes possibles qui mettraient les effets de l'eau en relation avec la théorie de l'adsorption multiples couches de BRUNAUER, EMMETT ET TELLER.

ZUSAMMENFASSUNG

1. Die wechselseitige Reaktion von Kasein und Glukose (1 Molekül per freie Aminogruppe des Kaseins) in "trockenem" Zustand wurde durch Bestimmung des freien Amino-Stickstoffs und Messung des Farbenwechsels geprüft.

2. Es zeigte sich, dass die Verlustgeschwindigkeit von Amino-Stickstoff von den folgenden Faktoren abhängt:

a) Sie wird stark von der Wasseraktivität im System beeinflusst, erweist sich am grössten, wenn der Wassergehalt 65–70% relativer Feuchtigkeit entspricht, fällt auf kleine Werte bei sehr hohem und sehr niedrigem Wassergehalt. Dieses Verhältnis zeigte sich bei 37, 70 und 90° C.

b) Sie steigt mit dem p_H-Wert an, von niedrigen Werten bei p_H 3, bis zu mindestens p_H 8, wahrscheinlich bis zu p_H 10.

c) Sie steigt progressiv an bei Temperaturzunahme von 0–90° C, und entspricht, wenn die relative Feuchtigkeit konstant bei 70% gehalten wird, der Gleichung von ARRHENIUS mit $Q_{10}^{15-25°\,C}$ von 5.4.

3. Farbenbildung ist gesteigert bei Zunahme von Wassergehalt, p_H und Temperatur.

4. Eventuelle Mechanismen zur Beziehung des Wassereffekts auf die vielschichtige Adsorptionstheorie von BRUNAUER, EMMETT UND TELLER werden erörtert.

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