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

IV. DECOMPOSITION OF THE AMINO-SUGAR COMPLEX AND THE REACTION OF ACETYLATED CASEIN WITH GLUCOSE

by

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INTRODUCTION

In previous communications from this laboratory[1-6] on the interaction between casein and glucose in the solid state it has been shown that the most rapid, and in the earliest stages virtually the only reaction occurring is a combination of glucose molecules with free amino groups of the protein, which consist mainly of the ε-amino groups of the lysine residues. No appreciable discoloration or loss of solubility occurs at this stage[1,2]. Subsequently, progressively more carbohydrate becomes attached to the protein than can be accounted for by combination with free amino groups, and other reactive side chains, including those of arginine and histidine and probably also of tyrosine and methionine, become involved[4]. Insolubility and a brown discoloration develop.

The situation, after the initial phase of the reaction, is obviously complex, with the possibility of protein groups reacting: a. directly with glucose, b. with reactive decomposition products of glucose or of the glucose-amino complex or c. by cross linking with carbohydrate already attached to the amino groups of the protein. A catalysed caramelisation of the sugar, followed by adsorption on to the protein is also possible, although few signs of such a process were observed in the previous work. In the present paper an attempt is made to throw further light on the nature of some of the chemical changes involved by:

1. A study of the behaviour of a casein-glucose complex isolated at an early stage of the reaction on further holding at 37° C in the absence of free sugar. Any reaction of carbohydrate already bound to protein amino groups (or of degradation products derived from such carbohydrate) with other reactive protein groups should be detectable by this means.

2. Measurement of the water produced in the reaction between casein and glucose, and in the degradation of the isolated amino-glucose complex, and

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3. Investigation of the reaction between glucose and a modified casein in which the free amino groups have been blocked so that the primary amino-glucose reaction cannot take place.

EXPERIMENTAL

1. *Decomposition of the amino-glucose complex at 37° C*

Material used

For purpose of studying the behaviour of the amino-glucose complex in the absence of free sugar the casein-glucose sample 5D previously described[4] was used. This material (which had been prepared by reaction of casein with glucose for 5 days at 37° C and 69% relative humidity in an atmosphere of nitrogen, followed by removal of the uncombined sugar) was practically white in colour and still freely soluble, but had lost 70% of its initial content of protein free-amino groups and contained 520 mg of bound carbohydrate per g total N. Only very minor proportions of amino acid side chains other than those of lysine had been affected[4].

Methods

Portions of the material were equilibrated at 10° C to moisture contents corresponding to a. 69% and b. 85% relative humidity at 37° C, sealed in glass ampoules under nitrogen, and held at 37° C for periods up to 3 months with examinations at intervals.

Solubility. For following changes in solubility 50 mg samples of the stored material (dry, sugar and ash-free casein basis) were mixed with 5 ml of water and a drop of toluene, and held at 25° C overnight. The p_H of the fluid was approximately 6.2. The mixture was then "tumbled" for a further 24 hours at 25° C by slow rotation of the tube containing a glass bead, centrifuged and the soluble N content of the supernatant fluid determined.

Dialysable solids. A 2% solution or suspension of a stored product was dialysed twice through cellulose for 2 days at 0° C into 10 volumes of distilled water and the combined extract evaporated in a rotary film vacuum evaporator (see appendix). The cellulose sac used contained traces of dialysable material even after exhaustive washing, and correction for a "blank" dialysis had to be made.

Amino-N. Changes in free amino-N content were followed by the VAN SLYKE procedure, using a reaction time of 30 minutes at 20° C, as previously described[2].

Arginine. Arginine was estimated by means of the specific arginine decarboxylase[7], after complete hydrolysis of the stored material with acid.

Colour. The colour of the solid was measured by reflected light using the Lovibond-Schofield Tintometer, as previously described[1].

Reducing power. Capacity to reduce potassium ferricyanide at p_H 5 was determined according to the method of CHAPMAN AND MCFARLANE[8]. Changes in the carbohydrate component of the casein-glucose preparation were also followed by the cuprometric method of SOMOGYI[9].

Results

The experimental results are collected in Fig. 1.

Solubility. Fig. 1A shows that insolubility developed in the 5D material on holding at 37° C, and that the process was more rapid at 85% R.H. than at 69% R.H., a marked induction period being present at the lower humidity. The development of insolubility, however, required about 80 days for completion in isolated 5D at 69% R.H. as compared with less than 30 days at the same relative humidity in the presence of excess glucose (cf. sample 30D, Fig. 1[4]). Only part of this difference is likely to be attributable to reaction of the 30% of residual free amino groups in 5D, leaving part still to be accounted for—perhaps by reaction of protein groups other than the amino groups with glucose.

Dialysable solids. During storage for 55 days at 69% R.H. *c.* 7 mg dialysable solids

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were produced from 1 g protein, corresponding to 58 mg/g N. If this dialysable material be assumed to derive from the carbohydrate attached to the protein it represents 11% of that initially present. It contained no nitrogen and exhibited no reducing power (cuprometric method).

Amino-N. It had been anticipated that a further fall in the residual free amino-N content of 5D might have occurred during storage, even in the absence of free glucose, as a result of cross linking or of possible reaction with labile decomposition products arising from degradation of the carbohydrate already attached to the bound amino groups. No such fall, however, was observed, but instead there was a slight initial rise followed by constancy (Fig. 1B). This small rise in apparent amino-N, in the absence of any regeneration of free glucose (c.f. above), is perhaps more likely to be an artefact due to the production of non-amino substances yielding nitrogen in the VAN-SLYKE determination[10] than a result of reversibility of the primary amino-glucose reaction.

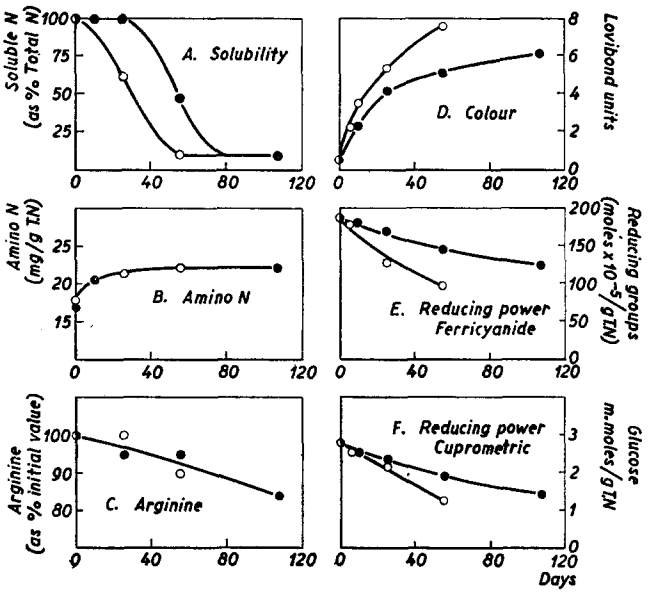

Fig. 1. Changes in casein-glucose complex 5D equilibrated to 69% R.H. (●) or 85% R.H. (◉) and held at 37° C in nitrogen in sealed tubes

Arginine. In the previous work[4] it was shown that amino acid side chains of several kinds, in addition to those of lysine, became involved in the later stages of the casein-glucose reaction, but nothing was discovered of the mechanism by which they did so.

Arginine was chosen for further investigation of this phenomenon because it reacted comparatively rapidly and was irreversibly destroyed in the sense that after reaction it could not be regenerated by the usual procedure for acid hydrolysis of the protein. Estimations by the specific decarboxylase method after acid hydrolysis of the stored 5D samples (Fig. 1C) showed a very slow disappearance of arginine at a rate which appeared to be independent of the relative humidity. Since no free glucose was present this slow destruction of arginine can only have resulted from 1. reaction with carbohydrate already attached to the protein amino groups or 2. reaction with decomposition products of the glucose-amino complex. In view of the persistence of the amino groups (Fig. 1B) the observed destruction of arginine would appear most probably to be due to mechanism (1) as postulated by FRAENKEL-CONRAT[11] for the protein-formaldehyde reaction. The rate of destruction of arginine in this way, however, was extremely slow, being only about one twentieth of that observed in the presence of free glucose[4].

Colour. Fig. 1D shows that degradation of the carbohydrate residue attached to the protein amino groups, as measured by the degree of browning, occurred more rapidly at 85% R.H. than at 69% R.H.

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After 25 days additional storage at 69% R.H. casein-glucose 5D developed a brown colour of 4.2 Lovibond units and this product, after admixture in solution with the required amount of glucose and re-drying, still showed a colour of 2.6 units as compared with the 3.9 units developed by casein which had been stored for the whole 30 days in the presence of excess sugar. Since only 70% of the casein amino groups had reacted in 5D as compared with 92% in the casein-glucose mixture at 30 days, it appears that, in the earlier stages of the reaction at least, the degradation of carbohydrate attached to the amino groups of the protein is able to account for most of the darkening in a casein-glucose mixture, and neither sugar bound to other protein groups nor the catalytic browning of free sugar plays a major part in discoloration. The presence of excess glucose, however, seems to influence the course of degradation of the amino-glucose complex since the proportion of the colour developed which is dialysable is less in the presence of glucose.

Ferricyanide reducing power. Previous experiments with milk protein[12] had shown that the power to reduce potassium ferricyanide at p_H 5 according to the method of McFARLANE[8] is associated with the protein-sugar reaction since it was not given by fresh or stored milk protein or lactose but increased with the loss of protein amino-N as milk powder deteriorated[13]. In the present work the dialysed casein-glucose preparation 5D was found to reduce ferricyanide strongly under the conditions of the McFarlane estimation, indicating that this is indeed a property of the amino-sugar complex.

Fig. 1E shows that the ferricyanide reducing power decreased as 5D was stored at 37° C at a rate which—like loss of solubility and development of colour—was greater at 85% R.H. than at 69% R.H.

Cuprometric reducing power. Direct determination of the total reducing power of 5D by the cuprometric method showed that its bound carbohydrate possessed about 90% of the reducing power of the free glucose from which it had been derived[2]. Since this proportion corresponded approximately with that part of the bound carbohydrate which was combined with the protein-amino groups the freshly prepared amino-glucose complex probably possessed a reducing power approximately equal to that of free glucose.

On storage at 37° C and 69% or 85% R.H. the cuprometric reducing power of 5D declined (Fig. 1F), disclosing a picture of progressive degradation somewhat similar to that shown by increasing colour and decreasing ferricyanide reducing power.

2. *Production of water in the casein-glucose reaction*

In previous papers[2,4] an indication was obtained that the average amount of water lost by glucose in becoming bound to casein was only of the order of one molecule of water per glucose residue in the early stages of the reaction, although it showed signs of increasing later. The method used however permitted only a rough estimate of the equivalent weight of the combined carbohydrate, and the point has now been re-investigated by direct determination of the increase in moisture content during the course of the reaction, employing preliminary drying at a relatively low temperature to minimise decomposition during completion of the process at a higher temperature. Changes in water content during the further storage of 5D in the absence of free sugar were also followed in order to obtain information on the behaviour of carbohydrate already bound to the protein, very largely at the amino groups.

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Methods

A mixture of sodium caseinate (p_H 6.3) with glucose (4.0 equivalents on the amino-N basis) was freeze-dried, adjusted at 10° C to a moisture content corresponding to a relative humidity of 69% at 37° C, and sealed in glass ampoules in an atmosphere of nitrogen. Moisture contents were determined initially and during subsequent storage at 37° C by drying *in vacuo* over magnesium perchlorate first at laboratory temperature and finally for 10 hours *in vacuo* at 70° C. Only *c.* 5% of the water content remained to be removed at the higher temperature. The disappearance of glucose from the reaction mixture was followed by means of notatin, and of free amino groups by the VAN SLYKE method. Bound carbohydrate was estimated from the increase in weight (or decrease in nitrogen content) of the undialysable solids.

Casein-glucose sample 5 D, without free glucose, was similarly stored and examined for moisture content.

Results

Fig. 2 shows that the quantity of volatile material (calculated as water) produced during the reaction between casein and glucose increased as glucose disappeared from the system. During the first few days the results could be accounted for almost entirely by reaction of the free amino groups each with a molecule of glucose, with the elimination of a molecule of water. Thereafter the liberation of water increased more rapidly than the disappearance of glucose, and at 80 days the molar ratio of water produced to glucose lost had reached 1.5.

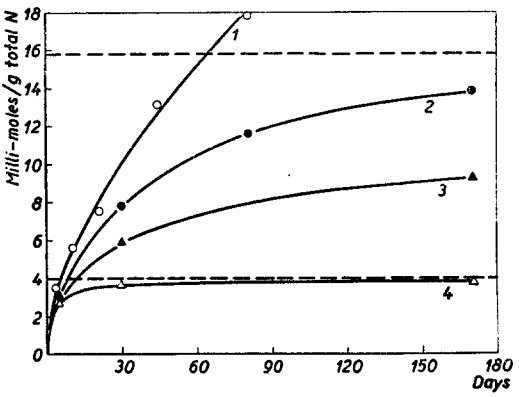

Fig. 2. Production of water during the reaction of casein with glucose at 69% R.H. and 37° C in nitrogen. 1 = Water produced; 2 = glucose reacted; 3 = Carbohydrate bound to the protein (molecular weight assumed 162); 4 = amino groups reacted. The dotted lines indicate the contents of amino-N and of glucose in the original mixture.

The assumption that the increase in volatile material was due almost entirely to the production of water was checked by comparison of the water content of the reaction mixture by titration with the KARL FISCHER reagent before and after storage for 30 days at 37° C. The water produced was found by this means to equal that indicated by the dry weight method. It has previously been shown[13] that only negligible amounts of carbon dioxide are produced from a milk protein-lactose mixture under nitrogen at 37° C and 55% R.H. (*e.g.* 0.02 millimol CO_2/g N in 30 days).

Casein-glucose sample 5 D stored under nitrogen in the absence of free glucose for 55 days at 69% R.H. or 85% R.H. produced no appreciable amount of water. Apparently, the considerable darkening in colour which occurred under these conditions (Fig. 1 D) was not associated with any very marked dehydration of the combined sugar. The small amount of water which might be expected from the slow condensation of arginine with carbohydrate (Fig. 1 C) would not readily be estimated by the method used.

3. *The reaction of acetylated casein with glucose*

OLCOTT AND FRAENKEL-CONRAT[14] have pointed out that acetic anhydride in limited quantity and in cold solution buffered by sodium acetate reacts fairly quantitatively and selectively with the free amino groups of bovine serum albumin, egg albumin and

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insulin. It was attempted therefore by this means to block the free amino groups of casein by conversion to the monoacetyl derivative, without too seriously interfering with other reactive groups or with the physical properties of the protein.

Although under none of the conditions investigated could a completely quantitative and selective acetylation of the amino groups be achieved, it was found practicable to block nearly all of the free amino groups of casein without introducing into the molecule more than a relatively few acetyl radicals in excess of those accounted for by mono-acetylation of the amino groups. The reactivity of the acetylated protein towards glucose was then investigated.

Methods

Acetylation of casein. In preliminary experiments sodium caseinate (p_H 6.3, 5 g) was dissolved in water (100 ml) and hydrated sodium acetate (38 g) added, followed by acetic anhydride (5.5 ml). Dropwise addition of the acetic anhydride during 30 minutes with vigorous stirring, or rapid addition during 1 minute followed by standing for 15 minutes or 5 hours, all gave approximately the same reduction of VAN SLYKE value, *i.e.* from 56.2 to *c.* 2 mg amino-N per g total N.

For larger scale preparations the acetic anhydride was added rapidly, and the reaction continued for 15 minutes, after which the gelatinous precipitate of acetylated casein was washed with water and finally freed from acetate ion by dialysis at 0° C. Total acetyl groups were estimated by refluxing with phosphoric acid followed by distillation and titration of the acetic acid in the distillates. Since no appreciable advantage in acetyl group/amino loss ratio was achieved by carrying out the reaction at 0° C, while the completeness of acetylation was perceptibly less, the material used for the storage experiments (Preparation 3, Table I) was acetylated at 10° C. Part of the small residual VAN SLYKE value of the acetylated product may well have been due to interference by substances other than amino-N, and acetylation can be considered to have blocked at least 94% and perhaps as much as 97% of the amino groups of the casein. Reaction with fluorodinitrobenzene however indicated that a few free amino groups were still present.

TABLE I

ACETYLATION OF CASEIN WITH ACETIC ANHYDRIDE

Preparation No.	Time and temp. of reaction	Amino-N (mg/g total N)		Loss of amino groups (mmol/ g total N)	Total acetyl groups (mmol/ g total N)	Acetyl group/ Amino loss ratio
		Casein	Acetylated Casein			
1	15 mins at 10° C	56.2	2.5	3.83	4.68	1.22
2	15 mins at 0° C	56.2	5.4	3.63	4.31	1.19
3	15 mins at 10° C	56.2	3.2	3.79	4.72	1.24

Preparation and storage of the acetylated casein-glucose mixture. On increasing the p_H of the acetylated casein from 5.3 back to 6.3 by addition of NaOH the protein redissolved and, after admixture with 4 equivalents of glucose (calculated on the amino-N content of the original casein), the product was freeze-dried from a 2% solution and equilibrated at 10° C to moisture contents corresponding to 70% R.H. at 37° C or at 60° C. At both temperatures the samples were held in sealed tubes under nitrogen.

Colour and ferricyanide-reducing power. These properties of the stored samples were determined as described above.

Arginine. Arginine was estimated on the intact material, without hydrolysis, by the colorimetric method of DUMAZERT AND POGGI[15] or, after complete acid hydrolysis of the protein, by the specific arginine decarboxylase[7].

Glucose. The disappearance of glucose from the reaction mixture was followed by the cuprometric method of SOMOGYI[9] applied directly and also after removal of the protein by precipitation with zinc sulphate and barium hydroxide.

Combined carbohydrate. Bound carbohydrate was determined directly as the increase in weight of the residue obtained after dialysis of the stored samples through cellulose at 0° C for 6 days in the presence of toluene, followed by freeze-drying and drying in vacuo for 3 days at 40° C and 3 hours at 70° C.

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Results

Colour. A brown colour developed in the acetylated casein-glucose mixture at 37° C and 70% R.H., but much more slowly than with casein-glucose under similar conditions, darkening after 30 days being 0.7 and 3.9 Lovibond units respectively in the two cases (Fig. 3A). This confirms the previous conclusion that in the earlier stages of the reaction at 37° C, at least, the free amino groups of the protein are largely responsible for the browning of casein-glucose.

At 60° C, however, discoloration developed approximately 25 times as rapidly as at 37° C, indicating that at the higher temperature the presence of free amino groups is not essential for rapid browning.

Arginine. Estimations of arginine in the acetylated casein stored with glucose at 37° C, by the decarboxylase method on the hydrolysate and by the less accurate colorimetric technique on the intact protein, both indicated a slow and linear rate of loss (Fig. 3B). As in the reaction of intact casein with glucose, arginine is destroyed irreversibly, in the sense that it cannot be regenerated by acid hydrolysis. The proportion of the initial arginine content of acetylated casein destroyed in 30 days, however, was only about 20%, as compared with 70% lost by casein under the same conditions. Apparently the destruction of arginine by glucose is accelerated by the presence of free amino groups in the protein, although it will proceed at a very appreciable rate in their absence.

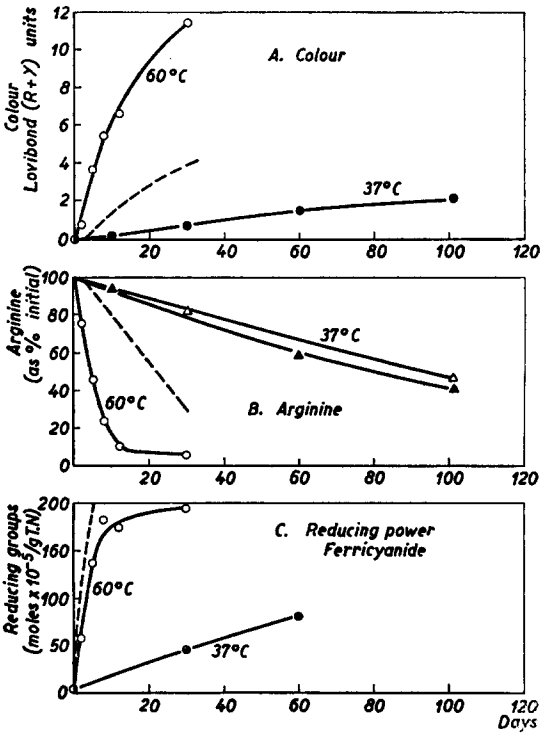

Fig. 3. Reaction between acetylated casein and glucose at 70% R.H. and 37° C or 60° C. The caseinglucose reaction at 37° C (dotted line) is included for comparison. Arginine was determined by decarboxylase in the hydrolysate (△) or by a colorimetric method in the intact protein (▲).

At 60° C the initial rate of destruction of arginine in acetylated casein by glucose was over 20 times as rapid as at 37° C, and 90% of the arginine content of the material had been destroyed in 12 days.

Ferricyanide-reducing power. The increase in ferricyanide-reducing power of acetylated casein stored with glucose, particularly at 60° C (Fig. 3C), shows that some other component of the system in addition to the amino-glucose complex is able to reduce ferricyanide under the conditions of the McFarlane test. To within the accuracy of the colorimetric methods the relationship between the ferricyanide reducing power and the disappearance of arginine is linear (Fig. 4), indicating that the arginine-glucose complex may be responsible for the observed reducing power.

Glucose. The disappearance of glucose from the reaction mixture at 37° and 60° C is plotted in Fig. 5. Since no consistent difference was detectable between glucose

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estimations carried out before and after removal of the protein by precipitation with zinc sulphate and barium hydroxide, it appears that glucose when it becomes bound to acetylated casein loses its power of reducing the copper reagent. This is in contrast to the observation with glucose bound to casein at the free amino groups, which displays a cuprometric reducing power approximately equal to that of free glucose (cf. page 369).

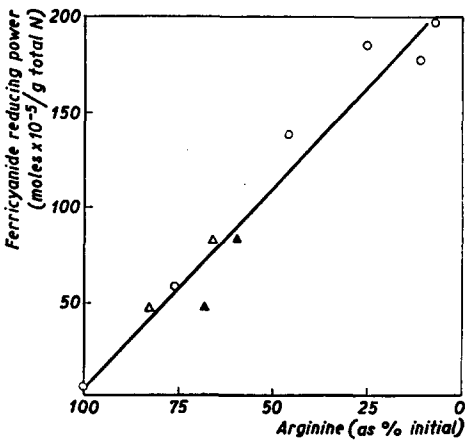

Fig. 4. Relationship between the ferricyanide reducing power of acetylated casein after reaction with glucose, and the disappearance of arginine. The symbols have the same significance as in Fig. 3B.

Combined carbohydrate. Determinations of the increase in weight of the undialysable (protein) fraction of two samples of the acetyl casein-glucose reaction mixture stored at 37° C, and of two samples stored at 60° C, are included in Fig. 5. The carbohydrate residue has been expressed as glucose on the assumption that one molecule of water has been lost per glucose molecule in combining with the protein. The results are sufficiently close to the amounts of glucose known by cuprometric titration to have disappeared from the reaction mixture to show that virtually all the glucose which so disappeared had become attached to the protein.

Inclusion in Fig. 5 of the arginine-loss values, calculated on the molecular basis,

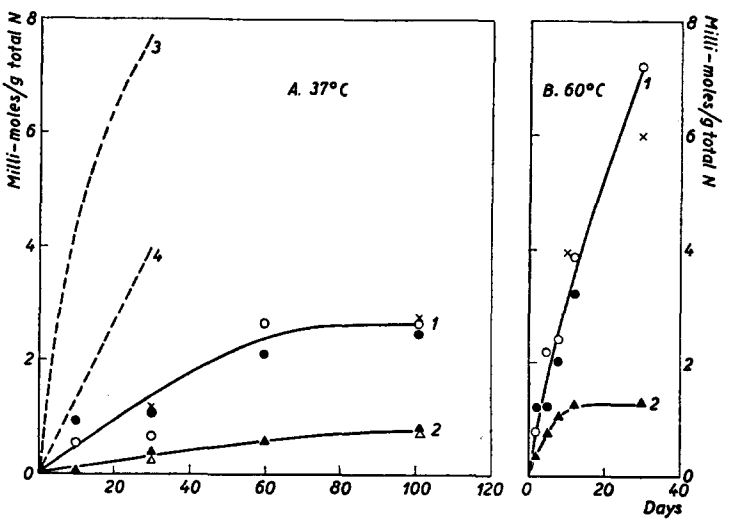

Fig. 5. Reaction between acetylated casein and glucose at 70% R.H. and 37° C or 60° C. Data for the casein-glucose reaction under the same conditions (dotted lines) are included for comparison with the 37° C results. The four symbols X indicate direct determinations of the amount of carbohydrate (equivalent weight assumed 162) combined with the protein.

A1, B1. Glucose reacting, as determined by the cuprometric method with (◉) or without (●) prior removal of the protein by precipitation.

A2, B2. Arginine reacting, as determined by decarboxylase (△) or by a colorimetric method (▲).

A3. Glucose reacting with casein.

A4. Glucose reacting with casein otherwise than with the amino groups.

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shows that the reaction of glucose with arginine (assuming a 1:1 ratio) can account for only about 25% of the initial rate of reaction of glucose with acetylated casein at 37° C and about 40% at 60° C. It has previously been shown (Table XIII[4]) that several other amino acid side chains in addition to lysine and arginine are involved in the reaction of casein with glucose at 37° C and 70% R.H. in proportions which would approximately account for such a result. Presumably these also react mainly with free glucose rather than with carbohydrate already attached to amino groups.

DISCUSSION

The experiments described in the present paper have been directed partly towards obtaining further information on the properties of the protein amino-glucose complex, and partly towards ascertaining whether further reaction of sugar already bound to amino groups or the attack of fresh glucose at other points of the protein surface is mainly responsible for the physical changes associated with deterioration and for the masking or destruction of amino acid side chains other than those of lysine.

It has been found that carbohydrate attached to the protein amino groups is comparatively unstable. Even at p_H 6.3, 37° C and 69% R.H. (or more markedly at 85% R.H.) it gradually decomposes (Fig. 1) with loss of its characteristic reducing properties, loss of solubility of the protein, and discoloration, this last developing sufficiently rapidly to account for most of the browning observed in a casein-glucose mixture under similar conditions of storage. Since comparatively little volatile or dialysable material was produced during 55 days at 69% R.H. decomposition of the carbohydrate under these (rather mild) conditions was limited in extent. Arginine disappeared at so slow a rate, compared with the casein-glucose system, as to suggest that secondary reaction of guanidyl groups with carbohydrate already bound to protein amino groups is of little importance in the dry casein-glucose system at 37° C. This behaviour is in contrast to that of the aqueous bovine serum albumin-glucose system recently investigated by MOHAMMAD, FRAENKEL-CONRAT AND OLCOTT[16], where it was concluded that guanidyl groups do not participate directly in the browning reaction at 53° C or below, and only become involved in secondary reactions when amino groups are available to initiate the primary reaction.

MOHAMMAD *et al.* found that at 53° C protein lacking appreciable amounts of free amino groups did not brown or undergo other types of reaction with glucose, although at 70° C browning occurred and some indication was obtained that other groups, possibly guanidyl groups, had reacted. In our experiments acetylated casein at 37° C and 70% R.H. definitely combined with glucose, although at only one third to one quarter of the rate shown by casein (Fig. 5A). A brown discoloration slowly developed and arginine was destroyed, again at one third to one quarter of the casein rate (Fig. 3B) and about four times as rapidly as it disappeared from the isolated amino-glucose complex. The rate of arginine loss (assuming a 1:1 combination with glucose) accounted for approximately one quarter of the total sugar reacting. This ratio corresponds to that previously found for casein[4] (after correction for the lysine not present in acetylated casein) where histidine, tyrosine and methionine were also shown to be reacting. Combination with glucose showed signs of slowing down before carbohydrate corresponding to the contents of all these acids had become attached to the protein (Fig. 5A).

At 60° C the reaction between acetylated casein and glucose was about 20 times

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faster than at 37° C, with rapid discoloration and rapid and complete destruction of arginine, despite the virtual absence of free amino groups from the system. The quantity of glucose combined with the protein after 30 days was approximately equivalent to the total content of the amino acids which had been found to be reactive in casein at 37° C and might well have exceeded that quantity subsequently. Since the main interest of the investigation was in more normal storage temperatures these observations at 60° C were not pursued further.

Carbohydrate bound to protein amino groups reduced both the SOMOGYI copper reagent and the MCFARLANE ferricyanide reagent. Carbohydrate bound to acetylated casein showed no cuprometric reducing power, although it reduced ferricyanide: there was some evidence that this latter property was due, in part at least, to carbohydrate attached to the arginine residues.

This series of experiments, on the whole, appears to support the hypothesis of direct reaction of the amino acid residues of the protein with glucose, rather than that of primary reaction of the glucose with amino groups (mainly in the lysine residues) followed by secondary cross-linking of carbohydrate so bound with other amino-acid side-chains. Certainly, the amino group-glucose reaction is much faster than any other, but in 5D the resulting product fails to react with arginine residues at a rate remotely approaching that required to account for the destruction of arginine in a casein-glucose mixture. Moreover, the large quantities of glucose which become attached to casein, and the low ratio of water produced to glucose reacted (1.28 at 30 days) both tend to be in conflict with a mechanism which requires extensive reaction of two amino acid residues with one molecule of glucose. Analytical data too have been produced[4] which indicate a ratio of 56.4 moles of lysine to 95.2 moles of arginine, histidine, tyrosine and methionine reacting, although the evidence for the participation of tyrosine and methionine is not sufficiently strong for reliance to be placed on this argument.

Unfortunately, the reaction of acetylated casein with glucose, which might have been expected to settle the question, failed to do so unequivocally. Acetylated casein undoubtedly reacted with glucose at 37° C, and arginine (and presumably other non-lysine side chains) entered into combination with the carbohydrate. They failed to do so, however, at a rate sufficiently rapid to indicate complete independence of amino groups in the casein reaction. This slowing down of the reaction of non-lysine side chains, typified by that of arginine, is believed more likely to be due to a depressing effect of acetylation (exerted possibly via steric or electrostatic factors or a changed R.H. optimum) than to the occurrence of any major proportion of cross linking in the casein-glucose reaction. However, the formation of occasional cross linkages, with resulting multiplication of the molecular weight and development of insolubility, as observed by MOHAMMAD *et al.*[16] with aqueous bovine serum albumin, is certainly not excluded by the experimental data.

The work described in this paper was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

SUMMARY

The decomposition of a casein-glucose complex in which the glucose was attached almost exclusively to the protein amino groups (70% of the total) was studied in conjunction with the reaction between acetylated casein (containing virtually no free amino groups) and glucose at 37° C and 69% or 85% relative humidity.

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The complex browned rapidly, at a rate which indicated that decomposition of carbohydrate attached to the protein amino groups could account for most of the darkening of a casein-glucose mixture at 37° C. The reducing power characteristic of the amino-glucose complex also decreased and insolubility developed, the change in each case being more rapid at 85% than at 69% R.H. Only a small proportion of the bound carbohydrate became dialysable, and practically no water was produced. Arginine disappeared extremely slowly, indicating that indirect reaction by cross-linking with carbohydrate already attached to amino groups was probably not an important factor in the casein-glucose system.

Acetylated casein stored with glucose browned only very slowly at 37° C, confirming the importance of free amino groups in this process. Arginine (and apparently other non-lysine side chains) reacted with glucose, although more slowly than in casein, and ferricyanide reducing power increased. At 60° C all three changes were accelerated about 20 times; very pronounced darkening occurred, much glucose combined with the protein and all the arginine groups were rapidly destroyed, notwithstanding the absence of free amino groups from the system.

In the early stages of the reaction of casein with glucose at 37° C and 69% R.H. approximately one molecule of water was liberated for each molecule of glucose reacting, the ratio subsequently rising to 1.28 after 30 days and 1.5 after 80 days.

RÉSUMÉ

Nous avons étudié, en conjonction avec la réaction entre la caséine acétylée (pratiquement sans groupes aminés libres) et le glucose, à une temperature de 37° C et une humidité relative du 69 ou du 85%, la décomposition d'un complexe caséine-glucose où le glucose était presque exclusivement attaché aux groupes aminés de la protéine (70% du total).

Le complexe brunissait rapidement, la vitesse du brunissement indiquant que la décomposition du hydrate de carbone attaché aux groupes aminés de la protéine pouvait bien être responsable pour la presque totalité du brunissement d'un mélange de caséine et de glucose à 37° C. Le pouvoir réduisant caractéristique du complexe amino-glucose diminuait également et on observa un développement d'insolubilité; le changement chaque fois était plus rapide au 85 qu'au 69% d'humidité relative. Une petite proportion de l'hydrate de carbone seulement devenait dialysable, et il n'y avait guère formation d'eau. La disparition de l'arginine était extraordinairement lente, à faire croire que la réaction indirecte par moyen d'enchaînements en travers avec l'hydrate de carbone déjà attaché aux groupes aminés n'était guère un facteur important dans le système caséine-glucose.

La caséine acétylée conservée avec du glucose brunissait seulement très lentement à 37° C, de façon à confirmer l'importance des groupes aminés libres dans ce procès. L'arginine (et, semble-t-il, d'autres chaînes latérales sans lysine) réagissaient avec le glucose, bien que plus lentement que dans la caséine, et le pouvoir réduisant le ferricyanide augmentait. A une température de 60° C, tous les trois changements étaient accélérés par 20 fois environ; le brunissement était très prononcé, une grande partie du glucose combinait avec la protéine et tous les groupes d'arginine étaient rapidement détruis en dépit de l'absence, dans le système, de groupes aminés libres.

Aux étapes initiales de la reaction de la caséine avec le glucose à 37° C et au 69% d'humidité relative, approximativement 1 molécule d'eau par molécule de glucose réagissant était libérée, proportion qui augmentait à 1.28 après 30 jours, et à 1.5 après 80 jours.

ZUSAMMENFASSUNG

Die Zersetzung eines Kasein-Glukose-Komplexes, in welchem die Glukose fast ausschliesslich den proteinischen Aminogruppen anhaftete (70% der gesamten Quantität) wurde in Verbindung mit der Reaktion von azetyliertem Kasein (welches praktisch keine freien Aminogruppen enthielt) und Glukose bei 37° C und 69 oder 85% relativer Feuchtigkeit untersucht.

Der Komplex wurde schnell braun, wobei die Geschwindigkeit des Braunwerdens darauf hinwies, dass das Dunkelwerden einer Kasein-Glukose-Mischung bei 37° C in der Hauptsache der Zersetzung des den proteinischen Aminogruppen anhaftenden Kohlenhydrates zuschreibbar ist. Das für den Amino-Glukose-Komplex charakteristische Reduktionsvermögen nahm auch ab und es entwickelte sich Unlöslichkeit, wobei die Veränderung in jedem Falle bei 85% relativer Feuchtigkeit schneller vor sich ging als bei 69%. Nur ein kleiner Teil des gebundenen Kohlenhydrates wurde dialysierbar, und es ergab sich praktisch kein Wasser. Das Arginin verschwand ausserordentlich langsam, was darauf hinwies, dass einer indirekten Reaktion durch kreuzweise Verkettung mit bereits Aminogruppen anhaftendem Kohlenhydrat wahrscheinlich im Kasein-Glukose-System keine grosse Bedeutung zukommt.

Mit Glukose aufbewahrtes azetyliertes Kasein wurde bei 37° C nur sehr langsam braun, ein Beweis für die Bedeutung der freien Aminogruppen bei diesem Vorgang. Das Arginin (sowie anscheinend auch andere Seitenketten ohne Lysin) reagierten mit Glukose, wiewohl langsamer als im Kasein, und das Ferrizyanid reduzierende Vermögen nahm zu. Bei 60° C beobachtete man eine etwa zwanzigfache Beschleunigung aller drei Veränderungen; das Braunwerden war auffallend stark, viel

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Glukose verband sich mit dem Protein, und alle Arginingruppen wurden trotz Abwesenheit freier Aminogruppen im System rasch zersetzt. In den Anfangsstadien der Reaktion des Kasein mit Glukose bei 37° C and 69% relativer Feuchtigkeit wurde ungefähr ein Molekül Wasser auf jedes reagierende Glukosemolekül befreit, während das Verhältnis nach 30 Tagen auf 1.28 angestiegen war und nach 80 Tagen 1.5 erreichte.

APPENDIX

A Modified Rotary Film Evaporator for Laboratory Use

In the work described in the present series of papers it has frequently been necessary to prepare acid-free protein hydrolysates by repeated vacuum evaporation, and to concentrate to small bulk at low temperatures large volumes of solutions obtained by dialysis or solvent extraction. For such purposes, and generally for rapid evaporation at reduced pressure without bumping or frothing the rotary film evaporator described by PARTRIDGE[17] has proved invaluable. A disadvantage of the original model, however, was that it could not be used with the acid vapours from protein hydrolysates which caused serious corrosion of the hollow metal spindle through which they passed.

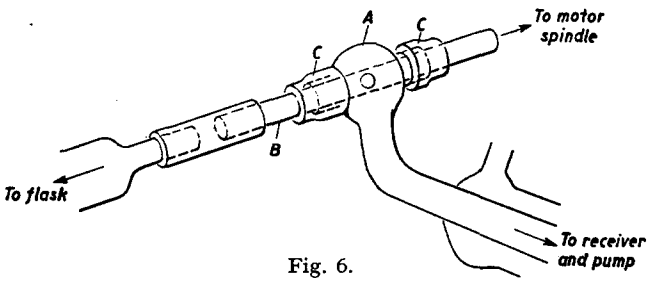

Fig. 6.

The design has therefore been modified to eliminate metal parts and at the same time simplified so that it can be assembled from ordinary laboratory glassware. A further modification permits collection of the distillate, which is usually essential in work with nonaqueous solvents.

The apparatus consists of a glass T piece A (Fig. 6), of which the cross piece of 11 × 9 mm tubing, expanded in the middle into a bulb, is clamped in a slightly inclined position. Through this cross piece passes a length of 8 × 6 mm tubing B, sealed at the upper end and carrying one or two holes in its side opposite the side limb of A. Two pieces of rubber tubing C fit the inner tube fairly tightly and are forced over the ends of A. The vacuum seal is thus obtained with easy rotation of the spindle B. Attached to the lower end of B by a short length of rubber tubing is a B19 cone on 15 cm of glass tubing. By means of standard adaptors any round bottomed flask of from 50 to 2000 ml capacity can be rotated on the water in a bath controlled at any desired temperature by a Simmerstat.

The drive is applied at the top end of B by attaching the glass tube to a stirrer spindle by a short length of rubber tubing. Any low speed, gear driven stirrer can be used, the Klaxon model recommended by PARTRIDGE being particularly suitable. The rubber glands are lubricated with a trace of Silicone stop cock grease and run for many hours in the presence of water or organic solvent vapours without trouble. They are easily replaced, and can even be run without lubricant if necessary. The side limb of the T piece leads from the underside of the bulb via a short water jacketed condenser to the detachable solvent receiver and metal water pump.

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