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

II. FURTHER OBSERVATIONS ON THE FORMATION OF THE CASEIN-GLUCOSE COMPLEX

by

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INTRODUCTION

In the first paper of this series[1] the effects of activity of water, of p_H and of temperature on the reaction between glucose and the free amino groups of casein were studied. A reaction mixture containing glucose and casein in the ratio of one molecule of glucose for each free amino group of the protein was used throughout, and comparisons of the rates of reaction were mainly confined to the earlier stages of the process in order to minimize interference due to side reactions. The effects observed on long storage remained relatively unexplored, among these being the fact that even under favourable conditions, namely at an activity of water corresponding to a relative humidity of 65 or 70%, the loss of free amino-N always slowed down or stopped when about 70% of the VAN SLYKE nitrogen initially present had disappeared: at lower activities of water the proportion was still smaller.

Attempts have now been made to carry the reaction more nearly to completion by simple physical changes in the system. The disappearance of the free amino groups of the protein has also been examined in greater detail by means of the fluorodinitrobenzene technique of SANGER[2], and information has been obtained regarding the number and reactivity of the ε- and α-amino groups of casein. A new attack based partly on the determination of glucose by use of the highly specific glucose oxidase (notatin) has revealed that the sugar is involved to an appreciable extent in changes other than the simple condensation with free amino groups. Finally, preliminary observations have been made on the behaviour of the protein-sugar complex to hydrolysis by dilute acid and alkali.

EXPERIMENTAL RESULTS

Effect of re-solution on the rate of the reaction

The observed failure of a proportion of the amino groups of casein to react with glucose might be due (a) to a lower chemical reactivity of these groups, (b) to steric

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factors dependent upon the configuration of the protein molecule or the prior introduction of the carbohydrate molecules already present or (c) simply to the limitation of access of the reactive groups to one another in the absence of sufficient moisture. If hypothesis (c) were correct re-solution of the reaction mixture after the rate had begun to slow down might be expected to increase the extent of the reaction.

Method

Sodium caseinate in 2% solution at p_H 6.3 was mixed with 1.5 equivalents of glucose (on the amino-N basis), frozen by rapid evaporation in glass tubes, and freeze-dried. The dried samples, after preliminary equilibration to the required moisture content were held at 37° C and 20 or 70% R.H. After 6 days under these conditions the contents of a number of the tubes were re-dispersed in water, freeze-dried, equilibrated and returned to 37° C for a further period. This process of re-dispersion and re-drying (which itself had no effect on the free amino content) was repeated once more after a total period of 12 days at 37° C. In this way three sets of samples of casein-glucose with 0, 1 and 2 re-dispersions were obtained, and the progressive loss of amino-N in each was followed by the VAN SLYKE method.

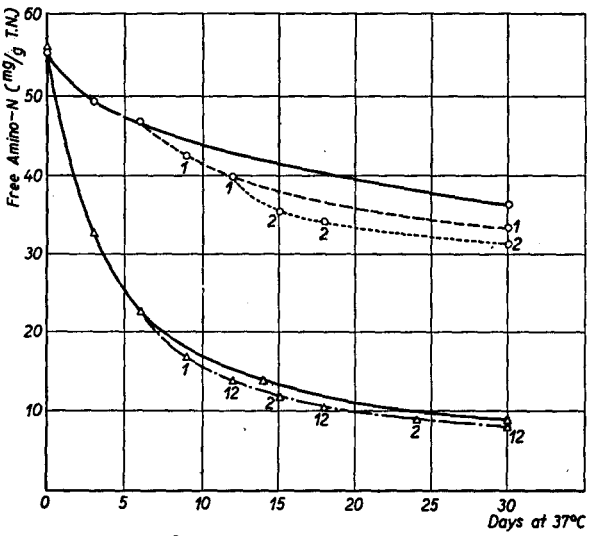

Fig. 1. Effect of re-solution after 6 and 12 days on the casein-glucose reaction at 20% (o) or 70% (△) R.H. The suffixes 1 and 2 indicate that the sample has been re-dissolved once or twice.

Results

The free amino-N values plotted in Fig. 1 show that, at 20% R.H. the simple process of re-dispersion of the solid in water and re-drying brings sugar molecules into positions from which they can attack other amino groups, and thereby progressively increases the total reaction. Calculation of the velocity constant of the reaction (assumed for this purpose to be of the second order) at the commencement of the experiment and immediately after the first and second re-solutions indicates no appreciable falling off in the rate of reaction, and shows that the $-NH_2$ groups which combine first with glucose are no more reactive than those attacked somewhat later.

When storage was at 70% R.H. the effect of the first re-solution process was very small, and of the second virtually nil (Fig. 1). It appears therefore that the slower reaction of the last one third of the free amino groups of the casein may be due rather to factors listed under (a) or (b) above than under (c).

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Effect of concentration of glucose on the rate and extent of the reaction

As a second possible means of increasing the extent of the reaction between protein amino groups and glucose, the effect of increasing the concentration of glucose was studied.

Method

Sodium caseinate-glucose mixtures containing from one to eight equivalents of glucose per free amino group were prepared as described above and held at 37° C and 70% R.H.

Results

From the results of the amino-N determinations (Table I) it can be seen that a considerable increase in the rate of combination of casein with glucose can be achieved by increasing the concentration of glucose up to about three equivalents, on the amino-N basis, but that little further improvement is obtainable beyond this point. Theoretical implications of these findings are discussed later (page 528).

The colour estimations (Table I) showed the usual initial 'lag' period during which one half to two thirds of the protein amino groups disappeared before perceptible discoloration occurred. The brown colour continued to increase in intensity, although somewhat more slowly, after the amino-aldehyde reaction had virtually ceased.

TABLE I

EFFECT OF CONCENTRATION OF GLUCOSE ON THE REACTION WITH CASEIN AT 70% R.H.

Number of equivalents of glucose	Free amino-N (as % initial value) remaining after days at 37° C					
	1	2	4	8	16	32
1	82	74	63	51	40	33
2	77	64	47	31	19	11
4	73	60	41	22	10	6
8	73	60	41	22	10	6
	Colour developed (Lovibond Y + R units)					
1	0.1	0.1	0.1	0.3	1.0	1.8
2	0.1	0.1	0.1	0.5	1.3	2.7
4	0.1	0.1	0.1	1.0	2.3	4.1
8	0.1	0.1	0.1	1.2	2.7	4.6

Investigation by the use of 1.2.4-fluorodinitrobenzene

A specimen of fresh sodium caseinate (C3) and two specimens of the casein-glucose complex, prepared as described below, were selected for a more detailed investigation by the FDNB method of the loss of protein amino groups. Samples 5D and 30D (Table II) had been stored for 5 and 30 days respectively and the unreacted sugar removed. They contained respectively 520 and 950 mg of bound carbohydrate per gram of total nitrogen, corresponding approximately to 7 and 13% of the weight of the dry material.

Method

The method was essentially as described by SANGER [2] and PORTER AND SANGER[3], and consisted of reaction of the proteins with the FDNB reagent, followed by hydrolysis of the DNP-proteins

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for 24 hours in 20% (w/w) HCl and examination of the hydrolysate by a chromatographic technique. The relatively long hydrolysis of the DNP-protein was found to be essential to ensure that all peptides were completely broken down, the labile DNP-glycine (estimation of which would demand a much shorter period of hydrolysis) having been shown to be absent by a preliminary examination of a 4 hour hydrolysate. No attempt was made to search for DNP-proline or DNP-hydroxyproline, the other labile derivatives.

The crude hydrolysate was extracted with ether and the aqueous and ethereal fractions run separately on silica gel partition chromatograms. The properties of the silica are critical; that used was prepared by Dr SANGER. The caseinglucose samples gave rise during hydrolysis to a considerable amount of humin, which remained almost entirely in the aqueous phase and was stationary on the columns used. A small amount of humin which occasionally passed into the ether phase could be readily separated from the DNP-amino acids on an ether column.

Results

The main results are summarized in Table II.

Free ε-amino groups of lysine. The fraction of the hydrolysate which on extraction with ether remained in the *aqueous* phase separated into two bands when run on 66% methyl ethyl ketone-ether and 15% butanol-chloroform columns. One of these bands, which was brown in colour, was fast on both columns and was discarded as an artefact. The other was the typical ε-DNP-lysine band (R 0.6 and 0.3 in the two solvents) and was collected, evaporated to dryness, dissolved in N HCl and compared colorimetrically with a standard. For this purpose a Spekker photoelectric absorptiometer with a purple colour filter (No. 601) was used.

TABLE II

DISTRIBUTION OF THE FREE AMINO GROUPS (a) IN FRESH CASEIN AND IN THE CASEIN-GLUCOSE COMPLEX

Protein	Total-NH_2 by VAN SLYKE determination (b)	Lysine ε-NH_2 by chemical analysis[4]	FDNB data				
			Lysine ε-NH_2 (c)	α-NH_2 groups			
				Band A	Band B (d)	Band C	Total
Casein (C3)	55.1	51.0	49.5 ± 1.0	0.45	0.32	0.35	1.12
Casein-glucose (5D)	16.7	—	18.0 ± 1.0	0.15	0.01	0.10	0.26
Casein-glucose (30D)	4.5	—	4.5 ± 0.5	—	—	—	—

a) As mg-NH_2 nitrogen/g protein total nitrogen
b) 30 minute values
c) Sum of ε-DNP-lysine + half bis-DNP lysine
d) One half of the measured figure: this band is bis-DNP-lysine

The figures shown in column 4 of Table II are based on readings obtained in this way, plus a small contribution from the ether phase as described below. For the native casein they are in good agreement within (the experimental error of the method) with published figures for direct chemical determination of the lysine content[4], and therefore none of the lysine ε-NH_2 groups can be unreactive to FDNB, as has been found to be the case in some proteins[5]. Moreover, a comparison of the figures in columns 2 and 4 shows that FDNB reacts with all the residual free amino groups of the casein-glucose complex available to the VAN SLYKE reagents, and that the extent of the reaction with glucose as indicated by the VAN SLYKE method is the same as that measured under the milder conditions of the FDNB technique (p_H 8.5).

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Free α-amino groups. The *ether* extract of the hydrolysate when run on a chloroform column showed three distinct bands (besides the usual artefact band which was rejected), the R values being: band A, 0.8; band B, 0.2; band C, stationary.

Band A when run on an ethanol-ligroin column was seen to be composite, containing at least three components.

Band B was identified as bis-DNP-l-lysine by its behaviour on 33% ether-ligroin and glycol-benzene columns, separating from DNP-glycine on the former and from bis-DNP-tyrosine on the latter, but from bis-DNP-lysine on neither.

Band C ran slowly on a 1% butanol-chloroform column; on a 3% butanol-chloroform column it gave an R value of 0.4 and appeared to be composite. It is possible that this band contained decomposition products of the DNP-amino acids in Band A.

In view of the complexity of the mixture and the relative weakness of the bands, detailed identification of the α-DNP-amino acids present was not attempted. The intensities of the corresponding bands in the native and deteriorated proteins (both after 24 hours hydrolysis) could however be compared colorimetrically, and these figures (columns 5, 6 and 7 of Table II) show that the α-NH_2 groups as well as the ε-NH_2 groups of casein have reacted with glucose, and that their reactivities are of the same order. If the reactivities had been exactly equal the figures for the 5D casein-glucose would have been A, 0.16; B, 0.04 and C, 0.12 (B being proportionately weaker owing to the lower probability of a terminal lysine molecule retaining *both* its amino groups free to react with FDNB), as compared with the observed values of 0.15, 0.01 and 0.10. The experimental values, moreover, are all likely to be slightly low owing to the considerable effect, on bands as weak as these, of adsorbtion losses on the silica gel — the effect being most marked with band B which was very faint.

On none of the columns used could any bands be separated which might correspond to lysine substituted on one $-NH_2$ group by a DNP-group, and on the other by glucose or its decomposition product.

The free α-amino groups of casein. It is possible to obtain from the experimental data a tentative estimate of the total number of free α-NH_2 groups in native casein, if two assumptions are made. The first, based on the observation[6] that the absorption spectra of all the DNP-amino acids so far examined show a pronounced maximum at 3500 Å, is that the extent of the absorption at this wavelength produced by each of the bands dissolved in 1% $NaHCO_3$ is a measure of the number of N-DNP linkages. (The O-DNP group from tyrosine which would interfere[6] was shown to the absent from the ether phase). The second assumption concerns the corrections to be applied for breakdown during the digestion with 20% HCl. After inspection of the figures for the percentages of the various DNP-amino acids remaining after hydrolysis[3], the following figures for 24 hours hydrolysis were adopted as reasonably representative: band A 65%; band B 85%; band C 60%. Figures incorporating these two assumptions can only be approximate, but the error is probably not large and the figures suffice to show that the α-NH_2 content of casein is very low — of the order of one group per 90000 molecular weight, which accounts for a reading of approximately 1.1 mg amino-N per g total-N, or *c* 2% of the total content of free amino-N.

The values shown for bands A and C in Table II are derived on the above basis; for band B they are half the experimentally determined figure. The other half of this latter figure represents ε-NH_2 groups which have been included in the value shown in column 4, as mentioned earlier. It should be noted that all approximations used in interpreting the experimental data have been applied equally to the control casein and to the casein-glucose samples, so that the ratio between the two —which represents the proportion which has reacted with glucose—will be unaltered.

A completely different sample of casein, prepared in the laboratory from fresh milk, gave the same results as the casein (purified from a commercial source) which was used in the above-described work. It appears likely, therefore, that the observed distribution of free α-amino groups is characteristic of the protein. No other investigations

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published to date give details of an examination of casein by the FDNB method, and the identification of lysine at the end of a polypeptide chain also appears to be unique.

Relationship between the numbers of protein amino groups and glucose molecules reacting

The results which have previously been reported with skim milk powder[7] and dialysed milk protein plus lactose or glucose[8] indicated that for each protein amino group inactivated during storage very approximately one molecule of sugar was bound to the protein. The nature of the substrates used and the unsatisfactory methods available for the determination of sugar combined with protein, however, prevented any close approach to accuracy in the determination of this ratio. The matter has therefore been re-investigated for the casein-glucose system using glucose oxidase (notatin) as a means of differentiating quantitatively between unchanged glucose and reducing substances other than glucose.

The properties of the enzyme have been fully described by KEILIN AND HARTREE[9], who showed it to be almost completely specific for glucose.

Methods

Sodium caseinate (p_H 6.3) was mixed in 2% solution with 1.0, 1.5 or 4.0 equivalents of glucose, freeze-dried, equilibrated to the required moisture content and held at 37° C and 70% R.H. Samples taken at intervals were examined as follows.

1. *Glucose by notatin.* The method employed was essentially that of KEILIN AND HARTREE[9], the oxygen absorbed being measured manometrically in the presence of ethanol and horse-liver catalase to ensure destruction of the hydrogen peroxide produced in the reaction. Neither protein nor any other component of the system interferred with the determination, although high concentrations of salts such as could result from the neutralization of acid hydrolyzates were objectionable. Calibrations were carried out with pure glucose under the conditions of the determination to check the activity of the enzymes. Glucose combined with casein was *not* oxidized by notatin, and samples of the casein-glucose complex containing as much as 18% of combined carbohydrate gave no reaction for glucose.

2. *Reducing substances (as glucose) by the cuprometric method.* 5 ml portions of solution containing approximately 1 mg of glucose were heated with 5 ml of the copper reagent of SOMOGYI[10] in a freely boiling water bath for 10 minutes. Free glucose and part of the combined glucose are oxidized under these conditions.

Determinations were carried out (a) directly on the casein-glucose sample, to give the 'total' reducing power, and (b) on the filtrate after precipitation of the protein by zinc sulphate and barium hydroxide (SOMOGYI[11]) to give the 'free' reducing power. The difference between (a) and (b) measured the 'bound' reducing power, *i.e.*, that precipitated with the protein. A second estimate of the bound reducing power was obtained directly (c) by the cuprometric method after removal of all 'free' reducing substances by dialysis through cellulose at 0° C. The presence of protein in determinations (a) and (c) decreased the sharpness of the end point, and casein itself showed a small apparent reducing power which was difficult to determine accurately. Calibration experiments with mixtures of glucose and protein in the range of compositions normally encountered indicated a recovery of 95% of the glucose added: a corresponding correction has therefore been applied to determinations by the cuprometric method in the presence of protein.

3. *'Bound' carbohydrate by weight.* The stored casein-glucose samples, after dialysis at 0° C to remove free sugar, were freeze-dried, and then dried *in vacuo* over anhydrous magnesium perchlorate (anhydrone) at 10° C, 37° C, and finally for several hours at 70° C. By removing practically all the water present before heating, decomposition during drying was largely prevented, and there was little or no change in weight at 70° C. From these data and the total nitrogen content of the samples the weight of the carbohydrate residue attached to the protein could be calculated.

Results

In preliminary experiments two casein-glucose mixtures (p_H 6.3) containing respectively (a) 1.0 and (b) 1.5 equivalents of glucose per free amino group were freeze-dried from a 2% solution, stored at 37° C and 70% R.H. and examined at intervals for loss of free amino-N and loss of free glucose. The results (Table III) showed that while

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during the first few days the losses of glucose and of amino-N were approximately equivalent, appreciably more sugar was lost subsequently than could be accounted for by the loss of amino-N, the discrepancy increasing with the duration of storage.

TABLE III

RELATION BETWEEN THE LOSS OF FREE AMINO-N AND OF FREE GLUCOSE IN CASEIN-GLUCOSE MIXTURES CONTAINING INITIALLY 1.0 AND 1.5 EQUIVALENTS OF GLUCOSE PER FREE AMINO GROUP

Initial concentration of glucose (equiv.)	Days at 37° C and 70% R.H.	Loss of amino-N (milliequiv./g T.N.)	Loss of glucose* (milli mol/g T.N.)	Loss of glucose / Loss of amino-N
(a) 1.0	2	1.13	1.12	0.99
	5	1.81	1.82	1.01
	10	2.32	2.48	1.07
	32	2.96	3.49	1.18
(b) 1.5	3	1.69	1.69	1.00
	6	2.41	2.49	1.03
	9 (1)	2.82	2.94	1.04
	12 (1)	3.02	3.25	1.08
	12 (2)	3.03	3.34	1.10
	15 (2)	3.18	3.75	1.18
	18 (2)	3.28	4.04	1.23
	24 (2)	3.39	4.25	1.25
	30	3.38	4.52	1.34
	30 (1)	3.46	4.57	1.32
	30 (2)	3.45	4.59	1.33

* Determined in expt (a) by notatin, and in expt (b) by the cuprometric method after precipitation of the protein

1) = re-dissolved once; 2) = re-dissolved twice

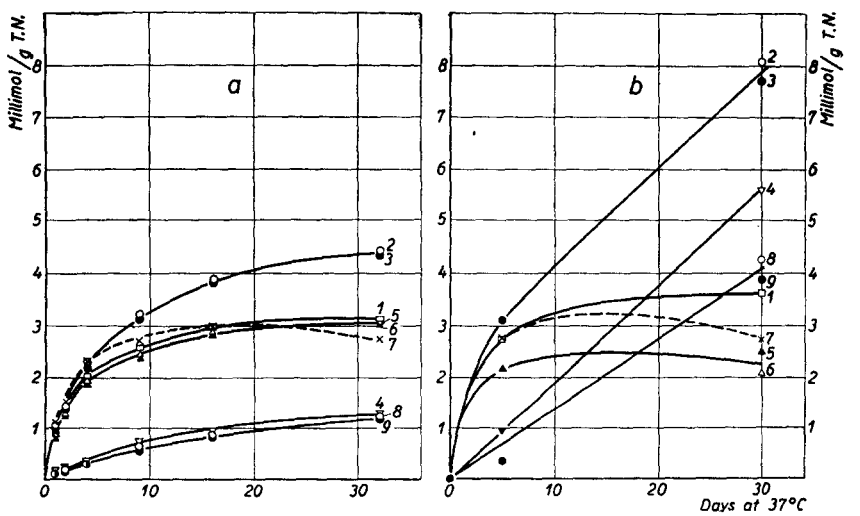

Fig. 2. Changes in the concentration of free amino-N and of glucose during the storage of casein-glucose containing (A) 1.5 and (B) 4.0 equivalents of glucose per amino group. 1. Loss of amino-N; 2. Loss of glucose (by notatin on the whole sample); 3. Loss of free reducing power (by the cuprometric method after clearing with zinc hydroxide); 4. Net loss of reducing power (by the cuprometric method on the whole sample); 5. Increase in bound reducing power (2–4); 6. Increase in bound reducing power (3–4); 7. Increase in bound reducing power (by the cuprometric method after dialysis); 8. Loss of glucose (notatin) other than by combination with amino groups (2–1); 9. Loss of free reducing power other than by combination with amino groups (3–1).

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The matter was therefore investigated in greater detail with results (Fig. 2) which may be summarized as follows:

a) The ratio between the rates of disappearance of glucose (curves 2 and 3) and of free amino-N (curve 1) approximated to unity in the early stages of the reaction but increased to 1.40 after 32 days, and 2.18 after 30 days in Figs 2A and 2B respectively.

b) A progressive loss of the 'total' reducing power of the system occurred during storage (curve 4), indicating conversion of some of the glucose to non-reducing or less strongly reducing forms.

c) The close agreement between curves 2 and 3 indicates that virtually all the reducing power remaining in solution after precipitation of the protein with zinc was unchanged glucose.

d) The three estimates of the increase in bound reducing power obtained (curves 5, 6 and 7) agreed reasonably well with the observed loss of free amino-N (curve 1) in Fig. 2A. In presence of the larger excess of sugar agreement was less good (Fig. 2B), possibly owing in part to error inherent in a result obtained by subtraction of two much larger quantities.

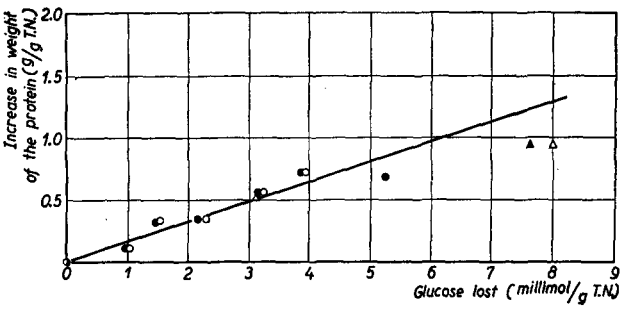

Fig. 3. The relationship between the quantity of glucose lost from a casein-glucose system and the increase in weight of the protein. Open symbols = glucose by notatin; solid symbols = glucose by the cuprometric method. Circles and triangles represent data obtained with glucose concentrations of 1.5 and 4.0 equivalents respectively. The line represents the addition to the protein of one carbohydrate residue of weight 162 ($C_6H_{12}O_6$–H_2O) for each molecule of glucose lost.

e) The progressive increase in weight of the undialysable fraction of the samples during storage (Fig. 3) showed that in both experiments nearly all of the glucose which had disappeared had become attached to the protein. Part of the sugar had therefore become attached to the protein otherwise than by combination with amino groups.

f) The fact that the loss of glucose (or reducing power) other than by combination with amino groups (curves 8 and 9) approaches in magnitude the net loss of reducing power of the whole sample (curve 4), suggests that the carbohydrate attached to the protein otherwise than by combination with free amino groups has little or no reducing power.

g) Direct determination of the 'bound' reducing power of the dialysed samples (calculated as glucose) gave values (curve 7) which, in the early stages of the reaction were equal to or even slightly in excess of the corresponding amino-N loss (curve 1). Apparently, glucose freshly bound to the amino groups has a reducing power towards the copper reagent of the same order as that of the free sugar. With continued storage however a gradual decline in reducing power occurred, probably associated with a certain amount of 'degradation' of the combined sugar.

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Preparation of bulk samples of the casein-glucose complex

For investigation of the properties of the casein-glucose complex two samples representing respectively a comparatively early and a late stage of the reaction were prepared in a quantity of several hundred grams, as follows.

Method

Casein, purified by re-precipitation at p_H 4.6 and solution in sodium hydroxide at p_H 6.3, was mixed with 4.0 equivalents of glucose and freeze-dried. For adjustment of the water content to a suitable value the dried product was agitated for 8 days in a revolving 10 litre glass vessel at 10° C while moist nitrogen was passed into the space above it. The moistened powder, which now contained water equivalent to a relative humidity of 68–69% at 37° C, was packed into a series of gas-tight vessels, in an atmosphere of nitrogen, and stored at 37° C part of it for 5 days and the remainder for 30 days. For the removal of unreacted glucose both products were taken up in distilled water and dialysed for several days at 0° C against water at p_H 8 (to prevent loss of sodium from the casein). After completion of dialysis the products (at p_H 6.3) were freeze-dried, adjusted to a water content corresponding approximately to 60% R.H. at 20° C, packed in a number of small moisture-proof vessels and held at —20° C until required.

The free amino-N contents of the 5D and 30D materials are given in Table II They contained respectively about 7 and 13% of 'bound' carbohydrate.

Hydrolysis by dilute acid and alkali

Since the properties of the protein-sugar complex in respect of its resistance to hydrolysis or decomposition by dilute acids and enzymes are of interest from the biological and nutritional points of view, a few preliminary investigations confined to attempts at recovery of complete sugar molecules from the complex by mild hydrolytic procedures are reported at this stage.

In previous (unpublished) work on milk powder and milk protein it had become apparent that the carbohydrate residue in the protein-sugar complexes formed in the dry state was attached to the protein much more strongly than in the reducing sugar-amino acid or peptide complexes formed in aqueous solution at slightly alkaline p_H. These latter apparently dissociate more or less completely with regeneration of the original reactants on acidification[12]. Treatment of a dialysed milk protein-lactose product with hot dilute acid had, in fact, failed to liberate much more free reducing power than would be expected from rupture of the lactose chain with liberation of galactose, leaving glucose attached to the protein.

Attempts to detect glucose after hydrolysis of the casein-glucose complex had perforce to be confined to conditions which would not bring about too great a destruction of free glucose. Parallel determinations with known quantities of glucose in the presence of the correct proportion of protein were therefore carried out side by side with those on the dialysed casein-glucose complex 5D. Free glucose was searched for, after hydrolysis of the complex, by treatment with notatin at p_H 5.6. N HCl at 100° C in an atmosphere of nitrogen was found to cause no liberation of glucose after 1, 2, 6 and 24 hours; decomposition of free glucose was 3% after 2 hours and 6% after 6 hours. In 0.1 N NaOH no glucose was liberated during 2 or 4 hours at 37° C, or 0.9 or 1.75 hours at 70° C: destruction of free glucose under these conditions was of the order of 40, 60, 75 and 95% respectively. It was not practicable to follow hydrolysis of the protein-sugar complex by amino-N determination, since treatment of casein itself with acid or alkali resulted in increases in the apparent VAN SLYKE-N, presumably owing to the hydrolysis of amide or peptide linkages.

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Although no free glucose could be regenerated by the treatment of the protein-sugar complex 5D with dilute acid or alkali, a considerable amount of soluble reducing substance was split off by the action of hot dilute acid. The 'total' reducing power shown in Fig. 4 was obtained by direct application of the cuprometric method to the product after hydrolysis and neutralisation. The 'free' reducing power was obtained after precipitation with zinc sulphate and barium hydroxide, and the 'bound' reducing power by difference.

The nature of the reducing substances split off from the casein-glucose complex by the action of hot dilute acid has not been determined. Examination of the products after hydrolysis for $^3/_4$ and 2 hours showed that no reducing substance fermentable by bakers' yeast was present, so that fructose as well as glucose was absent. Traces of furfurals, as detected by the SELIWANOFF reaction, were present.

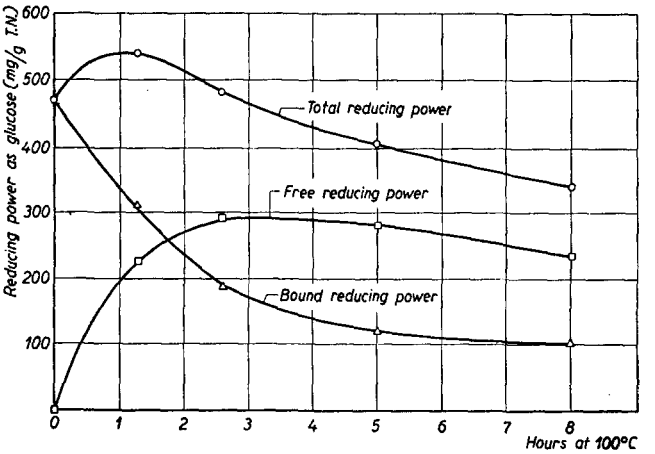

Fig. 4. Hydrolysis of the casein-glucose complex (sample 5D) by 2N HCl

DISCUSSION

The Van Slyke determination on casein and the casein-glucose complex

It has previously been shown[13] that the reaction between nitrous acid and casein or milk protein in the VAN SLYKE apparatus at 20° C proceeds at a very rapid rate during the first few minutes, and then falls away, merging after about 60 minutes into a very slow and approximately linear rate of increase. This slow increase was attributed to secondary reactions the effect of which could be eliminated by extrapolating the linear portion of the curve back to zero time or, approximately, by adding 2 mg amino-N/g T.N. to the 30 minute value.

With the demonstration that the concentration of free α-amino groups in casein is of the order of 1 mg amino-N/g T.N., and that all the ε-amino groups (51 mg amino-N/g T.N.) are free, it becomes apparent that the total concentration of free amino-N actually present in casein is approximately 52 mg amino-N/g T.N. This compares with 30 minute VAN SLYKE figures of 52–55 obtained on several samples of isoelectric casein or sodium caseinate p_H 6.3. On a sample of casein-glucose complex (5D) the concentration of free amino groups found by the FDNB method was approximately 18, as compared with approximately 17 by the VAN SLYKE (30 minute) method. On sample

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30 D the value was 4.5 by both methods, and extrapolation to zero time of the amino-N/time of reaction curve gave a value of approximately 5.0.

In view of these results the 30 minute VAN SLYKE value, without correction, has been taken in the present paper as representing the free amino-N content of the various experimental samples, although it is realized that for fresh or nearly fresh casein the result is likely to be slightly too high.

Physical aspects of the reaction between the amino groups and glucose

The present experiments provide further support for the conception of the reaction between the glucose and the free amino groups as taking place in a film of water on the surface of the protein, the film increasing with increasing relative humidity until it covers the surface in the region of 65% R.H. This interpretation, as already pointed out[1], is based on the observations of BULL[14] and accounts for the fact that, in a mixture containing one molecule of glucose per free amino group, the rate of loss of amino groups reaches a maximum at 65–70% R.H. The marked effect, now observed, of re-solution at 20% R.H. as compared with the negligible effect at 70% R.H. is obviously in agreement with such a picture, and a closer examination of the effect of increasing the concentration of glucose shows that this too is consistent. If, following BULL, each water molecule is assumed to occupy a surface area of 10.65 A^2 at 37° C, the amount of water known to be present at 70% R.H. and 37° C would, if in a unimolecular layer, cover an area of 0.56 sq. metres/mg of dry protein. It can then be seen that the concentration of glucose which produces a maximum reaction rate, *i.e.*, approx. 3 equivalents per amino group (33 g/100 g dry protein), could also cover this area with a unimolecular film if the area occupied by each glucose molecule was 50 A^2. This is in fact the average of the approximate figures of 40 to 60 A^2 for a glucose molecule with the plane of its ring respectively perpendicular to and parallel to the surface, as quoted by ADAM[15] from work with cellulose and its derivatives: the figures for a tilted molecule could lie anywhere between these limits. The conditions for maximum reaction would therefore seem to be those in which unimolecular layers of both water and glucose are present on the protein surface.

The nature of the union between the carbohydrate and the protein

From the data relating the loss of amino groups to the disappearance of glucose (p. 524) it appears that the reaction with free amino groups is the mechanism by which glucose combines most rapidly with the protein, and that this mechanism accounts for the greater part of the sugar destroyed in the early stages of storage, particularly when conditions are unfavourable for rapid reaction (*e.g.*, at low relative humidity) or when the quantity of glucose present is small. The fact that the compound formed by this reaction has a reducing power of the same order as that of free glucose, and the further observation that glucose cannot be regenerated from it by treatment with acid indicates that the product of the reaction between glucose and the free amino groups of casein is not a simple N-glycoside, although such a substance may well be first formed and immediately undergo isomerization by some intramolecular change such as the AMADORI rearrangement.

Under favourable conditions, *e. g.*, at 70% R.H., and in the presence of excess sugar, glucose continues to disappear from the system after the reaction with amino groups has virtually ceased. This extra glucose, comparable in amount with that combined

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with tha amino groups, is found also to be 'bound' to the protein, but the nature of the union with the protein has yet to be established with certainty. Alternative possibilities are (a) a chemical reaction between glucose and reactive groupings in the protein other than free amino groups, and (b) caramelization of glucose, catalysed in some way by the protein and followed by adsorption of the caramelized product on to the casein.

The colour of the darkest material obtained was only a light brown or 'biscuit' shade, and the average amount of water lost by the sugar in becoming 'bound' to the protein appeared to be only of the order of one molecule of water per glucose residue. This does not suggest any very extensive degree of caramelization. Furthermore, the 'extra' sugar held by the casein seemed to have little or no copper reducing power, while some, although not all of the simpler sugar anhydrides do possess reducing properties. Two attempts, made with hot methyl alcohol and cold 0.1 N sulphuric acid respectively, failed to elute any appreciable quantity of reducing material from casein-glucose complex containing 13% of 'bound' carbohydrate, of which approximately half was combined with amino groups.

In further work now in progress investigation is being made of the degree of destruction of individual amino acids as disclosed after complete hydrolysis of the protein by strong acid, and after digestion by proteolytic enzymes.

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SUMMARY

1. The reaction of the free amino groups of casein with glucose has been further investigated by VAN SLYKE determinations.

a) The extent of the reaction at a water content corresponding to 20% R.H. has been found to depend on the degree of hydration of the protein surface. Most of the individual amino groups appear to have the same reactivity.

b) At 70% R.H. the rate and extent of the reaction approach a maximum when three equivalents of glucose are present, this quantity approximating to that required to provide a unimolecular layer on the surface of the protein.

2. 1.2.4 fluorodinitrobenzene has also been used for estimation of the free amino groups.

a) It has been shown that all the ε-amino groups of the lysine side chains (51 mg amino-N/g T.N.) are free to react with the reagent, and that a small quantity of free α-amino groups (*ca* 1 mg amino-N/g T.N.) occur at the ends of polypeptide chains: these α-groups are derived from at least five amino acids, one of which is lysine.

b) The loss of amino-N during the reaction with glucose, as found by the VAN SLYKE method, has been quantitatively confirmed. Both the ε-amino groups and the α-amino groups have approximately the same reactivity towards glucose.

3. The rate of disappearance and the fate of the glucose have been followed by various procedures, including use of the specific glucose oxidase (notatin).

a) Combination of the free amino groups of casein, each with one molecule of glucose, has been confirmed as the most rapid and virtually the only reaction occurring at 20% R.H., and, in the earliest stages at 70% R.H. As storage proceeds at the higher humidity progressively more carbohydrate becomes bound to the protein than can be accounted for by combination with free amino groups: its weight indicates an average loss of one molecule of water per glucose molecule.

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b) The nature of the union between the extra carbohydrate and the protein is still uncertain. It may be a chemical union with reactive groups of the protein molecule other than amino groups, *i.e.*, with amino acid residues other than those of lysine. Alternatively, but less probably, it may be the result of caramelization of the glucose followed by adsorption of the product on to the protein.

c) None of the carbohydrate combined with (or adsorbed by) the casein can be oxidized by notatin.

d) Hydrolysis with dilute acid or alkali fails to regenerate any glucose (or fructose) from the casein-glucose complex, although other reducing substances including furfurals are liberated by the acid.

RÉSUMÉ

1. Nous avons poursuivi par les déterminations de VAN SLYKE, l'examen de la réaction des groupes amino libres de la caséine avec du glucose.

a) Etant donné un contenu aqueux correspondant à 20% d'humidité relative, nous avons constaté que la mesure de la réaction dépendait du degré d'hydratation de la surface protéique. La plupart des groupes amino individuels semblent posséder la même réactivité.

b) A 70% d'humidité relative, la vitesse et l'étendue de la réaction tendent vers un maximum, en présence de 3 équivalents de glucose, c'est-à-dire d'une quantité qui suffit à peu près pour obtenir une couche unimoléculaire sur la surface protéique.

2. Nous nous sommes également servis du 1.2.4 fluorodinitrobenzène pour estimer les groupes amino libres.

a) Nous avons démontré que tous les groupes ε-amino des chaînes latérales de lysine (51 mg amino-N/g N total) sont libres de réagir avec le réactif, et qu'un petit nombre de groupes α-amino libres (env. 1 mg amino-N/g N total) se trouvent aux extrémités de chaînes polypeptides, lesquels groupes α sont dérivés de 5 acides aminés au moins, dont un, la lysine.

b) Nous avons confirmé quantitativement la perte (constatée par la méthode de VAN SLYKE) de l'amino-N, pendant la réaction avec le glucose. Les groupes ε-amino et α-amino possèdent tous deux une réactivité à peu près identique avec le glucose.

3. Nous avons suivi la disparition du glucose et son destin à l'aide de procédés variés, dont l'emploi de l'oxidase spécifique du glucose (notatine).

a) Nous avons constaté que la réaction des groupes amino libres de la caséine, chacun avec une molécule de glucose, était la plus rapide et, en fait, l'unique réaction observable à 20% d'humidité relative, et aux étapes initiales, à 70% d'hum. rel. Quand la réaction progresse a l'humidité plus élevée une quantité de l'hydrate de carbone progressivement supérieure à celle correspondant à la combinaison avec les groupes amino libres, est liée à la protéine: le poids de celle-là suggère une perte moyenne d'une molécule d'eau par chaque molécule de glucose.

b) La nature la combinaison de l'hydrate de carbone en excès et de la protéine reste à éclaircir. Peut-être s'agit-il d'une combinaison chimique avec des groupes réactifs de la molécule protéique à l'exclusion des groupes amino, c.-à-d. avec des résidus d'acides aminés, à l'exception de la lysine. Ou bien, ce qui est moins probable, s'agit-il d'une caramélisation du glucose suivie d'une adsorption du produit sur la protéine.

c) L'hydrate de carbone combiné avec (ou adsorbé par) la caséine n'est pas oxydé par la notatine.

d) L'hydrolyse avec de l'acide ou de l'alcali dilués ne parvient pas à régénérer le glucose (ou le fructose) du complexe caséine-glucose, bien que d'autres substances réductrices, dont des furfurols, soient libérées par l'acide.

ZUSAMMENFASSUNG

1. Die Reaktion der freien Amino-gruppen des Kaseins mit Glukose wurde mit Hilfe von VAN SLYKE Bestimmungen weiter untersucht.

a) Das Ausmass der Reaktion bei einem Wassergehalt entsprechend 20% relativer Feuchtigkeit hing von dem Grade der Hydratation der Proteinoberfläche ab. Die meisten individuellen Aminogruppen scheinen die gleiche Reaktivität zu besitzen.

b) Bei 70% relativer Feuchtigkeit erreichen Geschwindigkeit und Ausmass der Reaktion ein Maximum, wenn drei Equivalente Glukose vorhanden sind, da diese Menge ungefähr ausreicht, eine monomolekulare Schichte an der Proteinoberfläche zu bilden.

2. 1.2.4-Fluorodinitrobenzol wurde auch zur Bestimmung von freien Aminogruppen verwendet.

a) Es wurde gezeigt, dass alle ε-Amino-gruppen der Lysin Seitenkette (51 mg Amino-N/g Gesamt-N) frei sind, mit dem Reagenz zu reagieren, und dass eine kleine Anzahl von freien α-Aminogruppen (*ca* 1 mg Amino-N/g Gesamt-N) an den Enden der Polypeptid-Ketten auftritt: diese α-Gruppen leiten sich von zumindest 5 Aminosäuren ab, von welchen eine Lysin ist.

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b) Der Verlust an Amino-N während der Reaktion mit Glukose, wie mit der VAN SLYKE Methode bestimmt, wurde quantitativ bestätigt. ε-Amino-gruppen und α-Amino-gruppen besitzen ungefähr gleiche Reaktivität gegenüber Glukose.

3. Das Mass des Verschwindens und das Los der Glukose wurde mit verschiedenen Verfahren verfolgt, einschliesslich der Verwendung der spezifischen Glukose Oxidase (Notatin).

a) Kombination der freien Amino-gruppen des Kaseins, jede mit einem Molekül Glukose, wurde bestätigt als die schnellste und eigentlich die einzige Reaktion, die bei 20% r.F. und, in den Anfangsstadien bei 70% r.F., auftritt. Mit fortschreitender Aufbewahrung bei der höheren Feuchtigkeit verbindet sich mehr Kohlenhydrat mit dem Protein als der Kombination mit freien Amino-gruppen entspricht: ihr Gewicht zeigt den durchschnittlichen Verlust eines Moleküls Wasser per Molekül Glukose an.

b) Die Natur der Verbindung zwischen dem Extra-Kohlenhydrat und dem Protein ist noch unbestimmt. Es könnte eine chemische Verbindung sein mit anderen reaktiven Gruppen des Protein Moleküls ausser den Amino-gruppen, z.B. mit andern Amino-säureresten als denen des Lysins. Anderseits, aber weniger wahrscheinlich, könnte es das Resultat sein von einer Karamelisierung der Glukose, mit nachfolgender Adsorption des Produkts an dem Protein.

c) Kein Teil des mit dem Kasein verbundenen (oder daran adsorbierten) Kohlenhydrats kann mit Notatin oxydiert werden.

d) Glukose (oder Fruktose) konnte von dem Kasein-Glukose Komplex durch Hydrolyse mit verdünnter Säure oder Alkali nicht zurückgewonnen werden, obwohl andere reduzierende Substanzen, einschliesslich Furfurale, durch die Säure freigesetzt wurden.

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