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

III. NATURE OF THE PROTEIN GROUPS REACTING

by

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INTRODUCTION

In Part II of this series[1] it was shown that in mixtures of casein and glucose at p_H 6.3, 70% relative humidity and 37° C the most rapid reaction occurring is that between glucose and the free amino groups of the protein (consisting mainly of the ε-amino groups of the lysine residues together with a few terminal α-amino groups). As interaction proceeds, however, progressively more carbohydrate becomes bound to the protein than can be accounted for by combination with free amino groups alone, and the purpose of the present paper has been to explore the possibility that amino acid side chains other than those of lysine might also be involved. Some indication of the existence of reactions of this kind has already been obtained in work with stored skim milk powder[2] where microbiological assay after enzymic hydrolysis showed that slight losses of histidine, arginine and methionine had probably occurred in addition to the much larger loss of lysine. HODSON AND KRUEGER[3] employing microbiological assay after acid hydrolysis also concluded that small losses of the same four acids had occurred in long-stored milk powders. Despite the apparent agreement between these findings the smallness of the changes observed and the known inaccuracies of the microbiological assay technique left the question of the participation of amino acids other than lysine in considerable doubt.

Working with an aqueous system PATTON, HILL, AND FOREMAN[4] have recently shown that refluxing casein with 5% glucose solution for 24 hours followed by acid hydrolysis and microbiological assay caused appreciable losses of lysine, arginine and tryptophan. With soyabean globulin under similar conditions some histidine also appear to be lost[5].

A serious difficulty in all investigations of this type has been the necessity for hydrolysing the protein before the amino acid groups involved in reactions with the sugar could be estimated. Treatment with strong acid or alkali at high temperature during such hydrolysis is not only liable to regenerate amino acids previously combined with carbohydrate, but may well destroy, by reaction with products of the degradation

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of carbohydrate, amino acids which had not previously reacted with the reducing sugar itself. Enzymic hydrolysis, while a little less open to the first of these objections and reasonably free from the second, nevertheless suffers from the additional disadvantage that it is usually far from complete, and the method of microbiological assay applied to the resulting mixture of free amino acids and peptides may be subject to errors.

Recently, ALBANESE[6] has claimed that certain chemical methods (mainly colorimetric) for the estimation of arginine, tryptophan, histidine, tyrosine, methionine and phenylalanine depend on reactive groupings still present when the acids are condensed to peptides, and are equally applicable to the estimation of these amino acids in free or peptide form. The peptides used by ALBANESE, however, were very small, and it is obvious that greater difficulties must be encountered in the application of such methods to the estimation of reactive groupings in intact proteins and especially in the insoluble products of the casein-glucose reaction. Nevertheless, the attempt has been made to achieve this result, although it is realized that the results obtained can be only of limited accuracy. In some cases, where the insolubility of a casein-glucose product prevented direct application of a chemical procedure, the material was first submitted to digestion by papain, such digestion however being limited to the smallest degree compatible with adequate dispersion in the reagents. For purpose of comparison the same amino acids have also been estimated after acid or alkaline hydrolysis of the protein by conventional procedures.

In addition to the reactive and chromogenic groups of the amino acids listed above, indications exist in the literature (*cf.* discussion) that amide and carboxyl groups of the protein might also be concerned in the reaction with reducing sugars, and estimation of these groupings in the intact (or as nearly intact as possible) protein and protein-sugar complex has therefore also been attempted.

EXPERIMENTAL

Materials

The preparation of the samples of casein and of the casein-glucose complex used for these experiments has already been described[1]. Briefly, they possessed the following properties.

Sodium caseinate (C3). Freeze-dried at p_H 6.3. Free amino-N content 55.1 mg/g total N. Glistening white soluble flakes.

Casein-glucose (5D). Prepared from sodium caseinate by reaction with 4 equivalents of glucose (amino-N basis) for 5 days at 37° C and 70% relative humidity in an atmosphere of nitrogen, followed by removal of the excess sugar by dialysis, and freeze-drying. Practically white in colour and still freely soluble. Free amino-N content 16.7 mg/g total N. Content of bound carbohydrate approximately 520 mg/g total N.

Casein-glucose (30D). Prepared as above but reaction allowed to proceed for 30 days. Light brown in colour and insoluble or sparingly soluble over a wide p_H range. Free amino-N content 4.5 mg/g total N. Content of bound carbohydrate approximately 950 mg/g total N.

Colour

5D was very pale cream in colour, and 30D a light biscuit-brown shade. Measured under standard conditions by reflected light in a Lovibond tintometer as previously described[7] the colours were C3 — 0.0; 5D — 0.1 red, 0.4 yellow; 30D — 1.3 red, 2.3 yellow units respectively.

Solubility

For comparison of the solubility of the three products at various p_H values, a quantity of casein or 5D equivalent to 15.65 mg N, *i.e.*, 100 mg of dry ash-free casein, after standing in water overnight, was treated with (a) 0.1 *N* HCl or 0.1 *N* NaOH and (b) 0.1 *N* NaCl, and made up to 5 ml with water, the total addition of (a) and (b) being 1 ml, corresponding to 0.02 *M* in the final solution. 30D gelled under these conditions, and was therefore made up to 10 ml; adjustment of the total ionic strength

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with sodium chloride was not attempted for the most acid and most alkaline samples. After tumbling slowly with glass beads at 25° C for 24 hours, the suspensions were centrifuged for 15 min at 3000 g and the soluble nitrogen content of the supernatant fluid determined.

The results obtained (Fig. 1) show that the solubility of the casein-glucose sample 5D was not detectably inferior to that of the original casein over the range tested, but that 30D had become very sparingly soluble over a wide pH range.

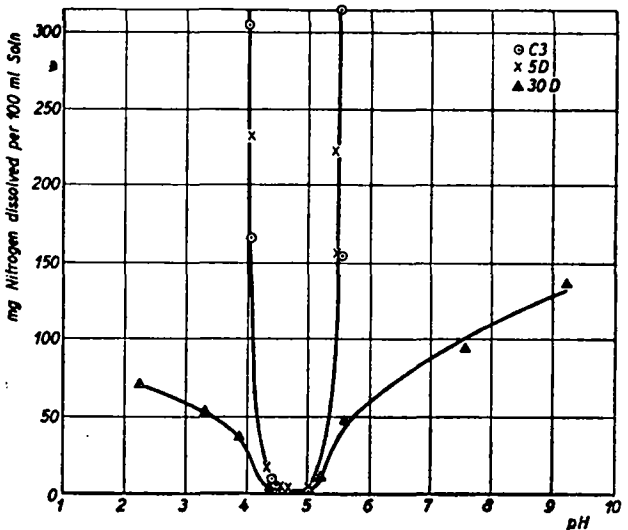

Fig. 1. Effect on the solubility of casein (C3) of reaction with glucose at 37° C and 70% R.H. for 5 days (5D) and 30 days (30D).

Estimation of amide groups

The amide groups of proteins are comparatively inert, and no means for estimating them in the intact protein appear to be available. Hydrolysis of primary amide groups is known to occur more rapidly than the splitting of peptide(secondary amide) bonds, but quantitative hydrolysis of the one without some hydrolysis of the other has not been achieved[8].

STEINHARDT AND FUGITT[9] employed hydrolysis at 65° C with a low concentration of a sulphonic acid of high molecular weight. The only material of this type available was a commercial sodium alkyl aryl sulphonate, and this was used at a concentration of 0.015 *M* in the presence of 0.05 *M* HCl. The liberation of ammonia however was very slow, and after reaction for 7 days only 65% of the amide-N had been released, with no significant difference between the three proteins.

A more conventional procedure was therefore adopted, whereby the proteins were refluxed with *N* HCl for various periods and the ammonia estimated by distillation with a slightly alkaline borate buffer. The results given in Fig. 2 show no indication of any difference in amide content caused by the reaction with glucose. If these groups do react with sugar they must be completely regenerated under the comparatively mild conditions of the amide estimation.

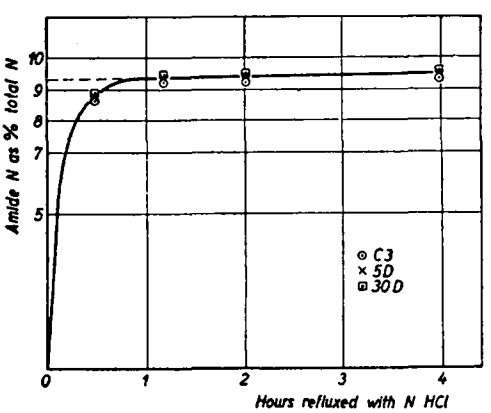

Fig. 2. The estimation of amide-N in casein C3 and in casein-glucose samples 5D and 30D.

Estimation of acidic and basic groups

Dye method

SCHMIDT[10] and others have utilized combination with dyes for estimating the acid and base combining properties of proteins. In the present experiments the general technique of FRAENKEL-CONRAT AND COOPER[11] has been followed, according to which the

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protein in buffered solution at p_H 11.5 is allowed to react with a basic dye (Safranin-O), or at p_H 2.2 with an acidic dye (Orange G), and the excess dye, after removal of the insoluble protein-dye complex, estimated colorimetrically. Safranin-O is a mixture of monacid bases of molecular weights 365 and 351; Orange G is dibasic, with a molecular weight of 453. The dye absorption method is said to be applicable to insoluble as well as to soluble proteins, provided that sufficient time is allowed for reaction. It has been claimed that Safranin-O combines stoichiometrically with the carboxyl, phenolic and sulphydryl groups of proteins, and Orange G with the guanidyl, amino and imidazole groups[11].

The results obtained with the basic dye (Table I) suggest that no loss of protein-acid groups occurs during the formation of the casein-glucose complex, a consistent small *increase* of the order of 10% being, in fact, observed with the 30D material. With Orange G also, little change was found, the 5D sample combining with approximately the same amount of dye as the control casein, and 30D with only 10% less.

TABLE I

ESTIMATION OF THE ACID GROUPS OF CASEIN AND CASEIN-GLUCOSE BY REACTION WITH SAFRANIN-O AT p_H 11.5*

Time of shaking (hours)	Concentration of dye (%)	Safranin bound (moles** per 10^5 g dry, sugar and ash free protein)		
		Casein (C3)	Casein-glucose (30D)	Difference
3	0.033	190	220	30
3	0.067	200	220	20
16	0.067	210	220	10
18	0.033	190	210	20
	0.067	200	220	20
	0.100***	180	200	20
	Average	195	215	20

* Protein concentration 0.04%
** Calculated on the assumption of a mean molecular and equivalent weight of 355.
*** Accuracy somewhat reduced by the large excess of dye.

Electrometric titration of acidic groups

Electrometric titration of a protein of comparatively low histidine content between p_H 6.0 and p_H 2.0 is considered to be a useful measure of the number of free carboxyl groups present[12], although any titration method is open to criticism because of the overlapping of the titration regions of the different groups. Theoretically, the best results should be obtained with concentrated protein solutions and in the presence of a large excess of a neutral salt, but in practice such conditions tend to produce insoluble coagula which fail to attain equilibrium with the aqueous phase in any reasonable time.

Casein (C3) and casein-glucose (30D) were titrated with HCl from p_H 6.0 to p_H 2.0 in the presence or absence of potassium chloride, the blank titration being carried out and the result calculated in terms of acid groups according to the method recommended

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by EDSALL[18]. Although the values obtained were not entirely independent of the method of titration employed they were consistent in again indicating no loss of acidic groups during formation of the casein-glucose complex, but rather a slight (*c.* 4%) increase (Table II).

TABLE II

"CARBOXYL" GROUPS* OF CASEIN AND CASEIN-GLUCOSE BY ELECTROMETRIC TITRATION BETWEEN p_H 6.0 AND 2.0

Protein** \ Conc. of KCl	0.0 *M*	0.1 *M*	0.2 *M*	Average difference
Casein (C3)	115	122	127	4
Casein-glucose (30D)	120	122	134	

* Equivalents per 10^5 dry, sugar and ash free protein.
** Initial concentration of protein 9–10%.

Titration of acidic and basic groups in aqueous acetone and alcohol

Titration in 90% alcohol or acetone with alcoholic KOH to thymolphthalein, and in 90–95% acetone with alcoholic HCl to naphthyl red has been used as a measure of the number of basic and acid groups in protein hydrolysates, and LINDERSTRØM-LANG and collaborators, using somewhat lower concentrations of alcohol or acetone, have applied the method to the determination of acid and basic groups in clupein[14], insulin[15] and β-lactoglobulin[16].

In the present experiments the proteins were titrated (a) with acid from p_H 6.0 to the naphthyl red end point in 70% acetone, and (b) with alkali from p_H 6.0 to the thymolphthalein end point in hot 80% alcohol, with the results shown in Table III. If we follow RICHARDSON[17] in assuming that the total acidic groups are titrated by (a), and the total basic groups minus arginine by (b), then it can be seen that the reaction between casein and glucose has caused no appreciable change in the number of basic groups present in the protein, but that a definite *increase* (*c.* 17%) in the number of acid groups again appears to have taken place.

TABLE III

ACIDIC AND BASIC GROUPS BY TITRATION FROM p_H 6.0 IN ACETONE AND ALCOHOL

Indicator	Solvent	Acidic or basic groups (equivalents/10^5 g dry sugar and ash free protein)		
		Casein (C3)	Casein-glucose (5 D)	Casein-glucose (30D)
a. Naphthyl red	70% acetone	108	121	126
b. Thymolphthalein	80% alcohol	105	107	106

Phosphate groups

Estimation of phosphate in the casein and in the casein-glucose samples 5D and 30D after digestion with perchloric acid gave the same result, indicating that no phos-

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phate had been split off from the casein and rendered dialysable during the reaction with glucose. No method was found for testing whether or not the phosphoric acid groups in the intact protein had reacted with glucose.

Estimation of individual amino acids

In investigating changes in several individual amino acid residues during formation of the casein-glucose complex the attempt was first made in each case to determine the content of the acid in the intact proteins. Where this failed owing to inadequate solubility of one or more of the protein samples in the reagents a minimal digestion with papain was resorted to. In order to detect any possible liberation of bound chromogenic groups under the action of the enzyme, samples were usually examined after several times of digestion. For purpose of comparison complete acid or alkaline hydrolysates were also assayed for each amino acid, and an attempt to assess destruction caused by the presence of carbohydrate during hydrolysis was made by hydrolysing casein and glucose mixed together in the proportions in which they had combined to form the casein-glucose complex 30D.

Digestion with papain

The protein in 2.5% aqueous solution or suspension was incubated at 70° C for the stated brief period with 2% of its weight of crude papain, added in warm aqueous solution after activation with a drop of potassium cyanide solution. Where necessary, HCN was removed in a current of nitrogen before the digest was used. A blank determination without protein corrected for the amino acid content of the enzyme preparation.

Acid hydrolysis

The proteins were refluxed with constant boiling HCl (35 ml per g protein) for 24 hours, and the excess acid removed by concentration twice *in vacuo* in a stream of nitrogen. The quantity of humin removable by filtration increased very markedly from casein, through 5D to 30D, although its total nitrogen content increased only slightly. After making up to volume the filtered hydrolysate was sealed in ampoules and held at —20° C until required.

Alkaline hydrolysis

The proteins were autoclaved for 7 hours at 121° C (15 lb pressure) with 5 *N* barium hydroxide (20 ml per g protein). The barium was precipitated as sulphate and the hydrolysate, after adjustment to p_H 7, was concentrated *in vacuo* and sealed in ampoules for storage at —20° C.

Lysine

In the proteins. The content of lysine in the intact protein was calculated from the amounts of ε-DNP lysine and bis DNP lysine recovered after treatment with fluorodinitrobenzene, followed by acid hydrolysis and chromatographic separation (*cf.* Part II[1], Table II). This reaction has the advantage of being carried out at p_H 8.5 and laboratory temperature, and decomposition of the protein-sugar complex under these mild conditions is very unlikely. An alternative estimate was made from the free amino-N content of the intact proteins, as determined by the VAN SLYKE method (30 minute reaction at 20° C), after correction for the small amount of terminal α-amino groups shown to be present by the FDNB method.

In hydrolysates. Lysine was estimated in acid hydrolysates of the three proteins by the specific decarboxylase method of GALE[18].

The results (Table IV) show that a rapid and extensive loss of lysine occurred early in the reaction with glucose, and that approximately 70% of this lysine was regenerated during the normal hydrolysis with acid for 24 hours. Continuation of acid hydrolysis

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for a further 24 hours failed to regenerate more lysine, any very small change being rather in the direction of a further destruction of the acid. No detectable destruction of lysine resulted from the hydrolysis of casein in the presence of added glucose.

TABLE IV

LYSINE§ CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Method	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	Fluorodinitro-benzene[19]	8.09 (100%)	2.94 (36%)	0.72 (9%)
	VAN SLYKE amino-N**	8.82 (100%)	2.86 (30%)	0.72 (8%)
	Mean	(100%)	(33%)	(9%)
Acid hydrolysate (24 hours)	Decarboxylase[18]	8.24 (100%)	6.69 (81%)	5.67 (69%)
Acid hydrolysate (48 hours)	Decarboxylase[18]	8.18 (100%)	6.27 (77%)	5.56 (68%)
Acid hydrolysate (24 hours) prepared in presence of glucose	Decarboxylase[18]	8.28 (100%)	—	—

* Literature value for the lysine content of casein 8.38 by a differential method, 8.25 by decarboxylase[20]

** Corrected for the small quantities of α-amino-N as estimated by FDNB.

Arginine

In hydrolysates. The SAKAGUCHI reaction with α-naphthol and hypochlorite or hypobromite in alkaline solution has been widely used for the estimation of arginine in protein hydrolysates, although opinions have differed considerably with regard to the conditions necessary to secure accurate results. ALBANESE[21] reduced the concentration of hypochlorite used from the 0.3 *N* of the original method to 0.06 *N*, claiming as a result higher colour stability and lower reagent blank. KEYSER[22] found it necessary to increase the hypochlorite concentration to 0.15 *N*. In our hands even this latter concentration of hypochlorite was inadequate for full development of the colour in protein hydrolysates and the concentration used was restored to the original 0.3 *N*. Under these conditions, with calibration against a chromatographically homogeneous sample of arginine, the acid hydrolysate of casein gave an arginine content approximating to the theoretical. The value, however, was not entirely independent of the size of sample taken and, since the accurate determination of arginine in crude hydrolysates appears to require a somewhat empirical and lengthy procedure[23] we have preferred to use for this purpose the specific decarboxylase method of GALE[18].

In the proteins. ALBANESE has claimed that the SAKAGUCHI reaction is applicable to small peptides[6], and has used the method without previous hydrolysis to establish the amount of protein present in body fluids[24]. KEYSER[22] also used the method for this latter purpose, but made no attempt to calibrate his results against an arginine standard.

§ All analytical data in this paper are given as g amino acid per 15.65 g protein-N, *i.e.*, per 100 g dry, ash and sugar free casein.

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No difficulty was experienced in obtaining consistent results by application of the SAKAGUCHI reaction directly to casein and casein-glucose without previous hydrolysis, even sample 30D dispersing adequately in the strongly alkaline reagent. The values obtained for casein by the ALBANESE technique, however, when calibrated against free arginine were very much higher than theoretical (Table V). The estimations were therefore repeated on all three proteins, making known additions of arginine to each and extrapolating back the straight line calibration curve thus obtained in presence of the protein. Again the same high values were obtained. The colour developed by the proteins was different (more pink) than that obtained with the free acid. Obviously, the chromogenic properties of the guanidine group in the protein and in the free amino acid were not sufficiently similar to permit calibration of the one against the other. Results obtained on the casein-glucose samples by this method must therefore be considered only as percentages of the values obtained for casein under identical conditions. These relative values were not seriously affected by variation in the concentration of hypochlorite used from 0.06 to 0.30 *N*, although the actual colours developed varied considerably.

TABLE V

ARGININE CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Method	Reagent	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	SAKAGUCHI[21]	NaOCl (0.30 *N*)	8.0 (100%)	6.8 (85%)	1.5 (19%)
Protein	SAKAGUCHI[22]	NaOCl (0.15 *N*)	14.2 (100%)	12.0 (85%)	3.0 (21%)
Protein	SAKAGUCHI[25]	NaOBr	3.63 (100%)	3.32 (92%)	1.06 (29%)
Acid hydrolysate (24 hours)	Decarboxylase[18]	—	3.85 (100%)	3.57 (93%)	1.18 (31%)
Acid hydrolysate (24 hours) prepared in presence of glucose	Decarboxylase[18]	—	3.79 (98%)	—	—

* Literature value for the arginine content of casein 4.04 by a colorimetric method[20], 3.85 by decarboxylase[28].

ROCHE AND MOURGUE[25] and MOURGUE[26] have recently applied the technique of DUMAZERT AND POGGI[27], which uses hypobromite with stabilization of the colour by alcohol, both to hydrolysates and to intact proteins, and have found lower values for the latter which they attributed to masking of some of the guanidine groups. Results obtained by this method on casein and casein-glucose (Table V) were more satisfactory than those obtained by the hypochlorite procedure, in that the arginine contents of the proteins as deduced by calibration against free arginine approximated quite closely to those obtained by the decarboxylase method on the acid hydrolysates. The values expressed as percentages of the casein value were not greatly different from the corresponding figures obtained by the ALBANESE technique.

The results (Table V) as a whole show that arginine also is involved in the casein-glucose reaction. It reacts much more slowly than lysine, but approximately 70% of the arginine side chains of the protein had been changed after 30 days. Since approxi-

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mately the same proportion of arginine was missing from the acid hydrolysate, and only a very small part of this loss appeared to be due to the presence of sugar during the hydrolysis, it must be concluded that the arginine side chains of casein which undergo reaction with glucose cannot be regenerated by the normal process of acid hydrolysis of the protein.

Histidine

In the proteins. For the estimation of histidine the method of ALBANESE, FRANKSTON AND IRBY[29], which is based on the PAULY diazo reaction was used. This method appears to have been employed previously only for hydrolysates and, by ALBANESE[6] for small peptides, but no difficulty was experience in applying it to casein and to the soluble casein-glucose sample 5D. The sparingly soluble casein-glucose sample 30D, however, gave a final coloured solution which contained a few coloured particles: this material was therefore treated with papain before use as described above. The colours obtained were compared with those from a chromatographically homogeneous sample of histidine, using an Ilford Spectrum Green filter (peak 520 mμ). The calibration curve was linear.

In hydrolysates. Histidine was estimated in the acid hydrolysates by the ALBANESE method, and by the specific decarboxylase method of GALE[18].

In hydrolysates of DNP-proteins. PORTER[31] has shown that the free imidazole groups of proteins react practically quantitatively with fluorodinitrobenzene under the usual conditions for the preparation of DNP-proteins, and that on hydrolysis histidine is *not* regenerated. Casein and the casein-glucose product 30D were therefore allowed to react with FDNB for 16 hours at p_H 8.5 and 25° C and the resulting DNP-proteins hydrolysed for 24 hours with constant boiling HCl. The histidine content of both hydrolysates, as estimated by the decarboxylase, was very small (Table VI) and no

TABLE VI

HISTIDINE CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Papain treatment	Method	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	—	Colorimetric[29]	3.22 (100%)	3.17 (98%)	2.06 (64%)**
Protein	3 min.	Colorimetric[29]	3.33 (100%)	—	2.35 ± 0.1 (68–74%)
Protein	6 min.	Colorimetric[29]	—	—	2.76 ± 0.2 (77–88%)
Protein	15 min.	Colorimetric[29]	3.37 (100%)	3.23 (96%)	2.78 ± 0.1 (80–86%)
Acid hydrolysate (24 hours)	—	Colorimetric[29]	3.27 (100%)	3.36 (103%)	2.72 (83%)
Acid hydrolysate (24 hours)	—	Decarboxylase[18]	3.12 (100%)	2.95 (96%)	2.23 (72%)
Acid hydrolysate (48 hours)	—	Decarboxylase[18]	3.09 (100%)	2.99 (97%)	2.26 (73%)
Acid hydrolysate (24 hours) in presence of glucose	—	Decarboxylase[18]	3.08 (99%)	—	—
Acid hydrolysate of the DNP protein	—	Decarboxylase[18]	0.10 (3%)	—	0.06 (2%)

* Literature value for the histidine content of casein 3.24[30], 3.18[30].
** Solution contained solid particles; result probably low.

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greater than would be expected from incomplete reaction of the FDNB with the protein, thus showing that all the imidazole groups of the original casein are free and that none of the histidine-"glucose" groups of the 30D is split by hydrolysis to yield free histidine.

The results collected in Table VI show that histidine has reacted in both 5D and 30D and is not recoverable by acid hydrolysis. The colorimetric figures for the acid hydrolysates of the deteriorated proteins are appreciably higher than those determined by the decarboxylase method, presumably owing to some difference in specificity of the reactions; the decarboxylase figure could be low owing to racemization or, more probably, the colorimetric figure could be high owing to the interference of unidentified degradation products. Assuming that the decarboxylase figure is the correct one and that no regeneration has taken place during hydrolysis we obtain values of 4% and 28% for the extent of combination of the histidine groups in the original protein-glucose complexes 5D and 30D respectively.

Confirmation by an alternative method is furnished by the series of results with the papain digests of the protein. Independent readings after any given period of digestion showed a fairly wide variation but there is a definite indication of an increase in recovery of apparent histidine as digestion proceeds, up to a figure equal to that already obtained with acid hydrolysates. The reason for this increase is unknown but an inspection of the results suggests that the correct figure for the histidine combined in the original intact 30D is 25–35%, which is in fair agreement with the previously obtained figure.

Tryptophan

The thymol method used by ALBANESE for hydrolysates[32] and small peptides[6] proved unsuitable for direct application to proteins, but more satisfactory results were obtained with the HORN AND JONES[33] modification of the *p*-dimethyl-aminobenzaldehyde method. This technique gave quite reproducible values for casein and the soluble casein-glucose complex 5D, but the sparingly soluble 30D again yielded a final coloured solution containing solid particles. After standing for 7 hours at 20° C, however, this turbid solution cleared, presumably owing to a hydrolysing action of the 8 *N* hydrochloric acid present. At this stage the colour was compared with that from casein, and from pure tryptophan (which gave a stable colour under these conditions) similarly treated. The results showed that if any tryptophan in the 30 day sample had been combined it had all been released by the time the product passed into solution. A similar result was obtained with papain: material digested for the minimum time necessary to bring about immediate solution in the reagents (3 mins) showed no significant loss of tryptophan. No difference could be detected between the tryptophan contents of the alkaline hydrolysates of the three proteins (Table VII).

Tyrosine

In the proteins. The modification of MILLON's reaction used by ALBANESE for the estimation of tyrosine in hydrolysates[34] or small peptides[6] is obviously inapplicable to proteins, but the procedure of ZUWERKALOW[35] which is carried out in concentrated acetic acid solution can be so applied. All the samples including 30D were soluble under these conditions. The calibration curve showed a marked curvature, and it was found necessary to work at almost exactly the protein concentrations recommended by ZUWERKALOW to obtain values for the tyrosine content approximating to the accepted

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TABLE VII

TRYPTOPHAN CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Extra treatment	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	—	1.62 (100%)	1.70 (105%)	—
Protein	8 *N* HCl, 7 hours at 20°C	1.37 (100%)	—	1.38 (101%)
Protein	Papain, 3 mins at 70°C	1.66 (100%)	—	1.59 (96%)
Alkaline hydrolysate	—	1.48 (100%)	1.47 (99%)	1.47 (99%)

* Literature value for the tryptophan content of casein 1.37[80]

literature values. The results obtained (Table VIII) show no significant difference between the casein, 5D and 30D.

The α-nitroso-β-naphthol method, although less accurate than procedures based on MILLON's reaction, is said to be more specific for tyrosine[36]. An attempt was therefore made to apply the technique of THOMAS[37] directly to the proteins, using an Ilford spectrum blue-green (peak 490 mμ) or green (peak 520 mμ) filter. A calibration curve deviating only just perceptibly from the linear was obtained with pure tyrosine.

Applied directly to casein and the soluble casein-glucose sample 5D the final coloured solutions contained a few minute, coloured particles: the sparingly soluble casein-glucose

TABLE VIII

TYROSINE CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Papain treatment	Method	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	—	MILLON[35]	7.19 (100%)	7.18 (100%)	7.51 (104%)
Protein	3 min.	α-nitroso-β-naphthol[37]	10.36 (100%)	9.13 (88%)	6.80 (66%)
Protein	7 min.	α-nitroso-β-naphthol[37]	10.30 (100%)	9.00 (87%)	6.24 (61%)
Protein	Mean	α-nitroso-β-naphthol[37]	10.33 (100%)	9.06 (88%)	6.52 (63%)
Acid hydrolysate	—	Decarboxylase[18]	6.30 (100%)	5.81 (92%)	5.19 (82%)
Acid hydrolysate prepared in presence of glucose	—	Decarboxylase[18]	5.83 (92%)	—	—
Alkali hydrolysate	—	MILLON[34]	6.35 (100%)	6.30 (99%)	6.14 (97%)
Alkali hydrolysate	—	α-nitroso-β-naphthol[37]	7.53 (100%)	7.07 (94%)	6.62 (88%)

* Literature value for the tyrosine content of casein 6.28[80].

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sample 30D showed more and larger particles. Despite this fault, which rendered quantitative assay impossible it was apparent that but little loss of chromogenic group had occurred in sample 5D, while in 30D the loss had been very considerable. After digestion with papain for 3 and 7 minutes the values recorded in Table VIII were obtained. Since these showed no indication of any progressive liberation of tyrosine during digestion with papain the mean of the two results has been taken as the best available indication of the proportion of the original phenolic groups remaining in the deteriorated proteins. Again, the chromogenic power of the phenolic residue in the protein was too different from that of the free amino acid to permit calibration, except against casein itself under similar conditions.

In hydrolysates. Acid hydrolysis of casein in the presence of glucose was shown by the specific decarboxylase method of GALE[18] to cause appreciable losses of tyrosine. Since losses observed with the casein-glucose samples were only of the same order of magnitude they were considered to be without significance.

It is not possible to examine alkali hydrolysates by the decarboxylase method owing to racemization of the amino acids. MILLON's reaction (ALBANESE-LUGG method[34]) showed no difference between the three samples, the α-nitroso-β-naphthol method showed only a small loss of doubtful significance.

The conclusion appears to be that the tyrosine side chains of casein react with glucose, and the resulting compound gives a colour with MILLON's reagent but not with α-nitroso-β-naphthol. The reaction is much slower than with the free amino groups, but after 30 days nearly 40% of the tyrosine had reacted. The product is largely split with regeneration of the tyrosine during the usual hydrolysis of the protein with acid or alkali, although in the former case part of the tyrosine is destroyed by humin formation.

Cystine

Most chemical methods for the estimation of cystine depend upon the reducing power of the reactive SH group and would be likely to be invalidated by the strongly reducing character of the carbohydrate present in the casein-glucose proteins. This amino acid has therefore been estimated only in the acid hydrolysates of the three proteins, and even with these it has been considered advisable to use the modified procedure of SULLIVAN AND HESS[38] as devised for urine, which also contains interfering reducing substances.

The results given in Table IX show that while definite losses of cystine are detectable in the 5D and 30D hydrolysates they are only of the same order as those obtained by hydrolysis of casein in the presence of glucose and are probably not significant. Cystine therefore does not react with glucose under the conditions of these experiments unless to form a complex which is very largely split during acid hydrolysis.

Methionine

The method of TOENNIES as used by ALBANESE for the estimation of methionine in hydrolysates[39] or small peptides[6] is based on the selective oxidation of methionine by hydrogen peroxide in the presence of perchloric acid, and cannot be expected to function in a substrate possessing the powerful reducing properties of the unhydrolysed casein-glucose complex. Even in acid hydrolysates reducing material of carbohydrate origin was still present since the casein-glucose complex 30D showed an apparent methionine content some 12% higher than that of the original casein.

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Attention was therefore directed towards the nitroprusside method of McCARTHY AND SULLIVAN as modified by HORN, JONES AND BLUM[40], but using a colour filter (Ilford spectrum green) corresponding more closely to the absorption band of the coloured solution[41]. Casein itself was precipitated by the concentrations of acid plus sodium chloride present in the final solution and even brief digestion with papain, as used in estimation of the other amino acids, would not hold the protein completely in solution at the concentrations required for the methionine estimation. Digestion with papain for rather longer periods gave the results listed in Table IX which show that methionine had reacted with glucose to the extent of nearly one quarter in 5D and over one half in 30D.

Difficulty was experienced in applying this colorimetric method and also that of CSONKA AND DENTON[41] to acid hydrolysates and, although the general indication was that the figures for the casein, 5D and 30D were similar, poor reproducibility made the results of doubtful value. The iodometric method of LAVINE as modified by POLONOVSKI AND ISSARTEL[42] however was found to be satisfactory and confirmed the observation that all the bound methionine was liberated during acid hydrolysis (Table IX).

TABLE IX

CYSTINE AND METHIONINE CONTENT OF CASEIN AND CASEIN-GLUCOSE

Amino acid	Determined in	Papain treatment	Method	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Cystine	Acid hydrolysate	—	Colorimetric[38]	0.34 (100%)	0.26 (77%)	0.25 (74%)
Cystine	Acid hydrolysate prepared in presence of glucose	—	Colorimetric[38]	0.28 (82%)	—	—
Methionine	Protein	16 hours	Colorimetric[40]	2.76 (100%)	2.10 (76%)	1.18 (43%)
Methionine	Acid hydrolysate	—	Iodometric[42]	2.63 (100%)	2.57 (98%)	2.50 (95%)

* Literature value for the cystine content of casein 0.40[30]; of methionine 2.51[40], 2.65[41], 2.90[42].

Phenylalanine

The method of KAPELLER-ADLER as used by ALBANESE[43] depends on the quantitative nitration and reduction of phenylalanine to the highly coloured ammonium salt of diaci-*o*-dinitrobenzoic acid. Inevitably, the rather drastic treatment with sulphuric and nitric acids must cause some decomposition of the protein-sugar complex, but no better method for direct estimation of the amino acid in the protein seemed to be available. Since neither the procedure used by KAPELLER-ADLER nor that used by ALBANESE for the suppression of interference by histidine could be applied to proteins no attempt was made to prevent interference by this acid or by tyrosine other than by the use of a filter with maximum absorption at 560 mμ as recommended by JERVIS *et al*[44]. Data given by ALBANESE indicate that under these conditions a result which is appreciably too high might be expected.

The results given in Table X show that the value obtained for the phenylalanine

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content of casein is in agreement with the higher of the values found in the literature. The still higher apparent phenylalanine contents of the deteriorated proteins are probably due mainly to interference by their combined sugar contents, as shown by the observation that admixture of glucose with casein in the proportions in which they are combined in 30D brought up the apparent phenylalanine content of the casein by 8%. There is no evidence in these data of any participation of phenylalanine in the reaction between casein and glucose.

TABLE X

PHENYLALANINE CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Protein	6.60 (100%)	7.03 (106%)	7.50 (114%)
Protein + glucose	7.12 (108%)	—	—

* Literature value for the phenylalanine content of casein 6.46[30], 5.50%[43].

Glutamic acid

Glutamic acid was estimated in the acid hydrolysates of the three proteins by the specific decarboxylase method of GALE[18], care being taken to minimize the formation of pyrrolidone carboxylic acid. The results (Table XI) show that there was no destruction of glutamic acid during the reaction of casein with glucose to form substances 5D and 30D. No destruction of glutamic acid occurred during the acid hydrolysis of casein in the presence of glucose.

TABLE XI

GLUTAMIC ACID CONTENT OF CASEIN AND CASEIN-GLUCOSE

Determined in	Casein* (C3)	Casein-glucose (5D)	Casein-glucose (30D)
Acid hydrolysate	22.2 (100%)	22.5 (101%)	22.1 (100%)
Acid hydrolysate prepared in presence of glucose	22.4 (101%)	—	—

* Literature value for the glutamic acid content of casein 22.1[30].

Effect of extending the reaction time to 164 days.

Summation of the amino acid losses in the 5D and 30D materials showed, as will be discussed later, that the totals are sufficient to account for a binding of carbohydrate in the ratio of one glucose unit per amino acid residue combined. It was therefore of interest to continue the reaction for a much longer period to ascertain whether saturation of the proteins with glucose could be achieved.

Method

The material was prepared under the same conditions as the 5D and 30D samples, *i.e.*, sodium caseinate mixed with glucose equivalent to four times the free amino-N content was maintained

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at 37° C and 70% relative humidity in an atmosphere of nitrogen for 164 days. The product was dark brown and almost granular in texture, the colour measured on the Lovibond tintometer being 4.8 red, 6.6 yellow and 0.5 "dullness" units.

The free glucose content was determined by use of the glucose oxidase notatin, and the weight of carbohydrate attached to the protein was estimated by dialysis at 0° C followed by dry weight determination.

Results

Table XII shows the results compared with those for 5D and 30D. The striking feature is that although most of the sugar which was still free after 30 days had disappeared after 164 days, only a little more than half of this had become attached to the protein. The remainder of the glucose had been converted into dialysable products the identity of which has not yet been investigated. Any loss of carbon dioxide or other gaseous product, or of water in excess of one mole per mole of carbohydrate, would also be included in this latter fraction, but could only represent a small proportion of the total.

TABLE XII

CHANGES IN DISTRIBUTION OF CARBOHYDRATE DURING THE REACTION BETWEEN CASEIN AND GLUCOSE AT 37° C, pH 6.3 AND 70% R.H.

Time of reaction (days)	Wt. of dry ashfree protein (g)	Free glucose (g)	Disappearance of glucose (g)	Carbohydrate attached to protein* (g)	Dialysable products (non-glucose) (g)
0	100	44	0	0	0
5	108	36	8	9	0
30	115	23	21	17	4
164	123	6	38	26	12

* Expressed as glucose, *i.e.*, increase in wt. of the protein $\times \frac{180}{162}$

DISCUSSION

Amide, acidic and basic groups

Amide groups. Interest in the determination of protein amide groups in relation to the protein-sugar reaction mainly lies in the observation of WORMELL AND KAYE and of FRAENKEL-CONRAT AND OLCOTT in the somewhat analogous protein-formaldehyde reaction. WORMELL AND KAYE[45] suggested that the acid hardening of casein in aqueous formaldehyde occurs largely by means of cross linking on to the amide groups by formaldehyde already attached to amino groups. FRAENKEL-CONRAT AND OLCOTT[46] concluded that the formation of cross linkages between amino and amide (or guanidyl) groups probably contributes greatly to the tanning or hardening action of formaldehyde on proteins at laboratory temperature over the range of p_H 2–9, and demonstrated by means of model systems that the primary reaction is the formation of methylol amines, not methylol amides. In the present experiments with casein glucose mixtures in the 'dry' state, it is abundantly clear that the free amino groups of the protein are heavily involved in the reaction with the sugar, but no evidence of any reaction involving the

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protein amide groups has been obtained. If the amide groups do combine at all under the conditions of the experiments the product must be quantitatively hydrolysed with regeneration of the free groups during the dialysis which removes excess sugar, or under the mildly acid conditions of the amide estimation.

Acidic groups. Acidic or 'carboxyl' groups in the casein and casein-glucose samples were estimated because the recent work of LEWIS[47] has shown that in aqueous solutions at temperatures in the region of 100° C the browning and caramelization of solutions of reducing sugars is greatly accelerated by the presence of carboxylic acids—hydroxy and other aliphatic as well as amino acids. During the course of the reaction carbon dioxide is evolved, in quantity corresponding to decarboxylation of the acid. In the present experiments on the casein-glucose system estimation of the free acidic or carboxyl groups in the protein, whether by adsorption of a basic dye, by electrometric titration or by titration with an indicator in an aqueous-organic solvent, all agreed in showing no loss of acidic groups during the reaction of casein with glucose. Slight *increases*, in fact, were observed in all cases probably owing to the formation of a few acidic groups in the carbohydrate residues attached to the proteins.

Confirmatory evidence that the free carboxyl groups of the protein had not been decarboxylated during the reaction with glucose was afforded by the glutamic acid estimations on the acid hydrolysates, which showed no appreciable difference between casein and the casein-glucose samples. Since the glutamic acid content of casein is more than three times the aspartic acid content and, moreover, the γ-carboxyl groups of the latter are probably blocked as amide groups in the intact protein[48], the free carboxyl groups of casein can be considered to be very largely those of glutamic acid.

While the reaction investigated by LEWIS may well be of importance in systems relatively rich in carboxylic acids and poor in amino compounds, such as fruits and fruit juices, there is at present no evidence that it plays any part in the reaction between protein and reducing sugar at low temperatures and approximately neutral p_H.

Basic groups. It has been shown in the sections dealing with individual amino acids that all three of the bases lysine, arginine and histidine become involved in the reaction of casein with glucose. General methods for estimating the basic groups in proteins might therefore have been expected to throw some light on the course of the reaction. Neither the dye method nor titration in aqueous-organic solvents, however, showed any appreciable reduction in the apparent number of basic groups present in the casein-glucose samples as compared with the original casein. It can only be concluded that the basic group-glucose combinations are still sufficiently basic in character to be estimated with the original groups. This conclusion is in agreement with the observation of ÅGREN[49], who found that a concentrated liver extract, rich in sugar and amino-N, lost free amino-N on heating or storage without any significant diminution in titratable amino groups. It would also explain previous failures to follow the amino group-reducing sugar reaction by means of the formol titration[2]. FRAENKEL-CONRAT AND COOPER[11] had previously observed that the treatment of proteins with formaldehyde caused only minor changes in the amount of the acid dye Orange G bound by them.

Individual amino acids

A 'balance sheet' summarizing the changes in the nine amino acids estimated in casein and in the casein-glucose products 5D and 30D is given in Table XIII. Results are included both for the proteins (either untreated or after minimal digestion with

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papain) and for the complete acid or alkali digests. The amounts of bound carbohydrate present in the two stored proteins are also shown. For ease of comparison the figures quoted are expressed as the number of residues in a hypothetical molecule of 100 000 molecular weight, the absolute values for the amino acid content of the control casein being taken as the figures determined on the hydrolysate, and the percentage figures for the unhydrolysed proteins converted to moles on this basis.

TABLE XIII

DESTRUCTION OR COMBINATION OF AMINO ACID RESIDUES IN RELATION TO THE QUANTITY OF GLUCOSE BOUND BY THE PROTEIN

Amino acid	Initial content in casein (C3)		Moles combined or destroyed/10^5 g dry casein			
			Estimated in protein		Estimated in acid hydrolysate	
	g/15.65 g N	Moles/10^5g*	5D	30D	5D	30D
Lysine	8.24	56.4	37.8	51.3	10.7	17.5
Arginine	3.85	22.1.	1.8	15.7	1.5	15.3
Histidine	3.20	20.6	0.6	5.8	0.8	5.8
Tyrosine**	6.32	34.9	4.2	12.9	2.1***	4.2***
Cystine	0.34	1.4	—	—	0.0	0.0
Methionine	2.63	17.6	4.2	10.0	0.4	0.9
Tryptophan	1.48	7.3	0.0	0.0	0.0***	0.0***
Glutamic acid	22.2	151.0	—	—	0.0	0.0
Phenylalanine	c. 6.6	c. 40.0	0.0	0.0	—	—
Total			48.6	95.7	15.5	43.7
Bound carbohydrate present, calculated as Moles glucose – H_2O (mol. wt. 162)			50	92	—	—

Total content of reactive amino acids (lysine, arginine, histidine, tyrosine, and methionine) in casein = 151.6 moles/10^5 g (153 if cystine be included). Bound carbohydrate found in 164 D = 142.2 moles/10^5 g, assuming a loss of 1 mol. of water per mol. of glucose.

* Dry, ash and sugar free basis, *i.e.*/15,650 g N.
** Determined by α-nitroso-β-naphthol; Millon's reagent indicated no loss.
*** Determined on alkali hydrolysates.

A potential source of error in these latter determinations is the possibility that the chromogenic properties of the group under investigation might be influenced by adjacent groups in the protein molecule, and might therefore be susceptible to changes in such groups due to reaction with glucose. Furthermore, it is always possible that some of the more labile protein-sugar links might be broken by the reagents used for estimation

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of the amino acids, although no evidence of such decomposition has been observed. Both of these difficulties are unavoidable with the methods available. The fact that all the free imidazole groups remaining in 30D were accessible to the relatively large FDNB molecule under very mild conditions suggests that error due to screening of the chromogenic groups by the carbohydrate molecules attached to the protein is not very serious.

Conclusions arising from these data may be summarized as follows:-

1. As previously reported[1] the reaction of the free amino groups of the protein (mainly the ε-amino groups of the lysine residues) with glucose is much the most rapid of the reactions occurring in the casein-glucose system. After 5 days at 37° C and 70% R.H. approximately two-thirds of the lysine side chains had combined with sugar. Small quantities of arginine, methionine and tyrosine, and probably traces of histidine, had also reacted, but the quantity of these acids taken together was still much smaller than the amount of lysine which had undergone reaction at that time. The lysine, arginine and histidine values were obtained on the intact protein, while for tyrosine and methionine minimal digestion with papain had to be resorted to in order to obtain the protein in solution.

The solubility and colour of the casein-glucose product at this stage of the reaction, when few amino acid residues other than those of lysine had been involved, showed practically no change from those of casein.

2. After reaction for 30 days over 90% of the lysine side chains had combined with glucose, and further change had become very slow. Approximately 70% of the arginine, 60% of the methionine, 40% of the tyrosine and 30% of the histidine residues had also reacted.

The values for lysine are based on two independent estimations on the intact protein, while those for arginine and histidine are derived from separate determinations on the proteins and on the acid hydrolysates. These figures should, therefore, be reasonably reliable. The data for methionine and tyrosine, however, depend solely on estimations on the protein carried out after minimal digestion with papain, and may well be less accurate. There was no evidence that amino acid-sugar linkages were split by the enzyme treatment.

Surprisingly, in view of PATTON's[4, 5] results with the aqueous casein-glucose system, no loss of tryptophan was found in these experiments. PATTON observed destruction of tryptophan after hydrolysis of the protein, either exhaustively with enzymes (for casein), or with alkali (for soy bean globulin). In our alkali hydrolysates of 5D and 30D no loss whatever of tryptophan had occurred, nor was any loss observable in 30D itself after the minimal treatment with acid or papain necessary to obtain the protein in solution. Obviously, the indole group of the tryptophan side chain does not react with glucose under our conditions, unless to form a very unstable complex.

3. The unreliability of attempting to estimate the number of amino acid residues which had been combined with sugar in the protein after complete hydrolysis of the protein-sugar complex by acid or alkali can be seen by comparing the appropriate columns of Table XIII. About 70% of the lysine which had been combined with glucose was regenerated during the usual acid hydrolysis procedure, the remainder being irreversibly destroyed since hydrolysis with acid for a further 24 hours failed to liberate any of it. All the methionine and most of the tyrosine which had been combined in the protein were recovered during acid hydrolysis; the remainder of the tyrosine was

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presumably lost in the formation of humin. Practically all of the tyrosine, however, could be recovered after hydrolysis with alkali. No loss of cystine was demonstrable after acid hydrolysis but, as with methionine it may well be that some part of the cystine reacted with glucose to form a link which is split during acid hydrolysis. Since cystine is only a very minor constituent of casein, clarification of this point by enzymic digestion of the protein was not attempted.

The case was quite different with arginine and histidine, where there was no regeneration of combined amino acid on hydrolysis with acid. This conclusion was confirmed for histidine by the independent fluorodinitrobenzene method. For these two acids, therefore, examination of the hydrolysates gives a reasonably accurate estimate of the extent of their combination with sugar in the intact protein.

Reaction mechanism

The mechanism of the reactions involving the amino acid residues other than those of lysine is still not clear. FRAENKEL-CONRAT, in a series of papers on the action of formaldehyde on proteins, concluded that formaldehyde combined with the free amino groups to give amino-methylol groups which then formed stable cross links by reaction with amide groups or with the reactive end groups of arginine[46], or of histidine, tyrosine or tryptophan[50]. Some of these cross linkings were intermolecular, and resulted in molecular aggregation and loss of solubility[51]. Under the same mild conditions (p_H 3–8) amines alone reacted to give amino-methylol compounds which, however, were unstable and could be readily decomposed by dialysis. The other groups alone did not react directly with formaldehyde under these conditions, but did so at elevated temperatures or in more acid or alkaline solutions.

It is tempting to speculate that some similar series of reactions takes place in the casein-glucose system. Certainly the free amino groups react most rapidly with glucose, and the other reactive protein groups subsequently involved are—with the notable exceptions of amide groups and tryptophan—substantially the same as those concerned in the protein-formaldehyde system. Moreover, the formation of cross linkages would explain the insolubility, even in guanidine hydrochloride or sodium dodecyl sulphate solution, which develops as these secondary groups disappear.

A number of features, however, are difficult to reconcile with a simple theory of uncomplicated cross linking involving only carbon 1 of the glucose.

1. Even when free amino groups are virtually the only constituent of the protein which has reacted with glucose the latter appears to have undergone some intramolecular change (such as an AMADORI rearrangement) as evidenced by failure to recover glucose on hydrolysis and by the strong reducing powers of the complex. The new reactive centres thus formed, together with any which might appear on further deterioration of the glucose, are potentially capable of forming cross links but the mechanism would be of a different type from that in the simple formaldehyde system.

2. The amount of glucose attached to the protein can become much greater than that required for a one to one reaction with the free amino groups alone. In fact at 30 days it is nearly sufficient to provide a separate molecule for each molecule of amino acid shown to have disappeared, and at 164 days the same relationship will hold assuming that the reaction of these amino acids has proceeded to completion. Whether the glucose has in fact combined molecule for molecule with all the amino acids affected has yet to be determined. The observed effect could be produced if part of the sugar

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had combined with carbohydrate molecules already attached to the protein, or if products of glucose decomposition or "caramelization" had simply been adsorbed by the protein.

3. The dialysable products (non-glucose) shown to be present in increasing amounts at and above 30 days could arise from spontaneous decomposition of glucose attached to the protein, or from breakdown of uncombined glucose induced by small amounts of reactive decomposition products. In either case, however, the products of breakdown are presumably aldehydic in nature and capable themselves of combining with and inactivating reactive amino acid end groups in the protein.

It must be left for future work to elucidate the precise nature of the combination between glucose and the various amino acids, the reason for the instability of the glucose, and the nature of the dialysable products.

In conclusion, reference must be made to the recent work of EVANS AND BUTTS[52, 53] who autoclaved soy bean protein alone and in the presence of sucrose, and submitted the product to microbiological assay after acid or enzymic digestion. They found that (a) lysine, aspartic acid and glutamic acid residues combined with some other constituent of the protein, probably free NH_2 with free COOH groups, to form enzyme-resistant linkages; (b) methionine, cystine and histidine side chains reacted with sucrose to form linkages resistant to enzymic but not to acid hydrolysis, while lysine and arginine formed linkages resistant to both. Reaction (a) does not appear to occur under the much milder conditions of the present experiments, nor does sucrose react with casein. Presumably partial inversion of the sucrose occurred during the autoclaving treatment used by EVANS AND BUTTS. A number of the features of reaction (b) appear to be common to both systems, although in the present work the greater part of the bound lysine was recoverable after acid hydrolysis whereas histidine was not. Tyrosine, which was not estimated by EVANS AND BUTTS, appears to react in the 'dry' casein-glucose system.

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SUMMARY

The disappearance of amino acid groups in casein has been followed after reaction with glucose at 37° C, p_H 6.3 and 70% relative humidity.

a. After 5 days approximately two thirds of the lysine had disappeared but other amino acids were only slightly affected.

b. After 30 days over 90% of the lysine had reacted and losses of other amino acids were now considerable. Approximately 70% of the arginine and 30% of the histidine had reacted, and about one half of the methionine and one third of the tyrosine had probably also disappeared.

c. Acid hydrolysis recovered all of the combined methionine, and two thirds of the combined lysine, but none of the arginine or histidine. Alkaline hydrolysis liberated most of the tyrosine.

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d. There was no demonstrable loss of tryptophan, phenylalanine, total acidic, total basic or amide groups.

e. Cystine and glutamic acid, estimated after acid hydrolysis, showed no change.

2. The weight of glucose which became attached to the protein was sufficient to provide one molecule for each molecule of amino acid which had disappeared after 5 and 30 days and probably also after 164 days, but it has not been proved that the reaction actually proceeds by reaction of one glucose molecule with each amino acid residue inactivated.

3. After 164 days approximately one third of the total glucose undergoing change had been converted into dialysable decomposition products.

4. Possible mechanisms of the reactions are discussed.

RÉSUMÉ

1. Nous avons examiné la perte de groupes d'acides aminés dans la caséine après réaction avec du glucose à 37° C, à pH 6.3 et à humidité relative de 70%.

a. Après 5 jours les deux tiers environ de la lysine avaient disparu tandis que les autres acides aminés n'étaient que légèrement atteints.

b. Après 30 jours plus de 90% de la lysine avaient réagi et les pertes d'autres acides aminés étaient fortes. Environ 70% de l'arginine et 30% de l'histidine avaient réagi, et vraisemblablement environ la moitié de la méthionine et le tiers de la tyrosine avaient également disparu.

c. Par l'hydrolyse acide la totalité de la méthionine combinée et les deux tiers de la lysine combinée ont été retrouvés mais point d'arginine ou d'histidine. L'hydrolyse alcaline a libéré la plupart de la tyrosine.

d. Aucune perte de tryptophane, de phénylalanine ou de groupes acides, alcalins ou amides n'a été démontrée.

e. La cystine et l'acide glutamique, dont la détermination a été effectuée après l'hydrolyse acide, restaient inaltérés.

2. Le poids du glucose combiné avec la protéine correspondait à une molécule de glucose à raison de chaque molécule d'acide aminé disparu après 5, après 30 et probablement aussi après 164 jours. Nous n'avons pas prouvé que la réaction a lieu, réellement, par la réaction d'une molécule de glucose avec chaque résidu inactivé d'acide aminé.

3. Après 164 jours environ le tiers du glucose transformé avait été changé en produits de décomposition dialysables.

4. Nous discutons les méchanismes possibles des réactions.

ZUSAMMENFASSUNG

1. Das Verschwinden von Aminosäure-Gruppen in Casein nach Reaktion mit Glucose bei 37° C, pH 6.3 und in 70% relativer Feuchtigkeit wurde untersucht.

a. Nach 5 Tagen waren ungefähr zwei Drittel des Lysins verschwunden. Andere Aminosäuren waren hingegen nur wenig angegriffen.

b. Nach 30 Tagen hatten über 90% des Lysins reagiert. Die Verluste an andern Aminosäuren waren beträchtlich. Ungefähr 70% des Arginins und 30% des Histidins hatten reagiert und ungefähr die Hälfte des Methionins und ein Drittel des Tyrosins waren wahrscheinlich gleichfalls verschwunden.

c. Durch saure Hydrolyse wurden das ganze gebundene Methionin und zwei Drittel des gebundenen Lysins wiedergefunden, aber kein Arginin oder Histidin. Alkalische Hydrolyse setzte den grössten Teil des Tyrosins frei.

d. Verluste an Tryptophan, Phenylalanin, sauren, basischen und Amido-Gruppen waren nicht nachweisbar.

e. Der Gehalt an Cystin und Glutaminsäure, welcher nach saurer Hydrolyse bestimmt wurde, zeigte keine Veränderung.

2. Die Glucosemenge, die an Eiweisskörper gebunden wurde, entsprach einer Molekel Glucose für jede Aminosäuremolekel, die nach 5, nach 30 und wahrscheinlich auch nach 164 Tagen verschwand. Der Nachweis, dass die Reaktion tatsächlich durch Reaktion einer Glukosemolekel mit je einem inaktivierten Aminosäurerest vor sich geht, wurde nicht erbracht.

3. Nach 164 Tagen war ungefähr ein Drittel der gesamten Umwandlungsprodukte der Glucose in dialysierbare Zerfallsprodukte übergegangen.

4. Der mögliche Reaktionsmechanismus dieser Vorgänge wird erörtert.

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