THE ACTION OF SULPHURIC ACID ON GLIADIN: WITH SPECIAL REFERENCE TO THE N-PEPTIDYL→O-PEPTIDYL BOND REARRANGEMENT[1]

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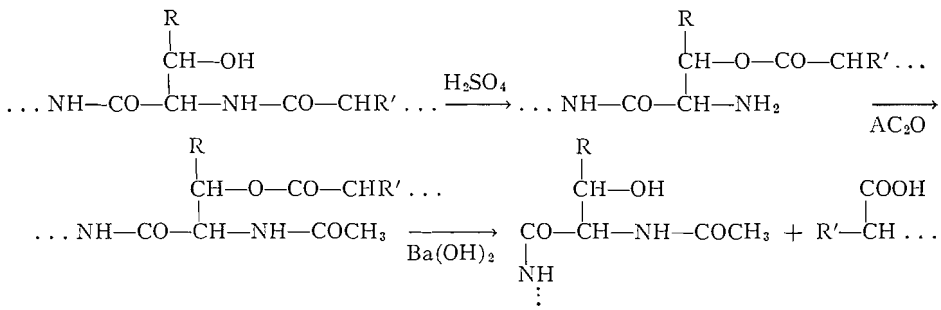
ABSTRACT

Treatment of gliadin with sulphuric acid transposes peptide bonds of serine from the amino to the hydroxyl group. Maximum transposition, 60–70% of the theoretical, occurs when the protein is treated with anhydrous sulphuric acid at 0°C. for 35 hr. No rearrangement was detected at threonine residues. Examination of the peptide material, obtained from the rearranged protein by Elliott's degradation method, indicates apparent "homogeneity". In an alternative scheme for the degradation, nitrous acid deamination of free amino groups was used. The resulting loss in serine content of the protein is direct evidence for the acyl migration of peptide bonds. Incorporation of sulphur and partial disappearance of several amino acids accompany the sulphuric acid treatment. The occurrence of these secondary reactions imposes limitations on the use of sulphuric acid as a reagent for the specific fission of peptide bonds.

INTRODUCTION

Attention has recently been directed towards methods which effect fragmentation of protein molecules in a predictable manner. Especially significant is Elliott's procedure for the cleavage of peptide bonds (7, 8, 9) at the amino groups of the β-hydroxyamino acids, serine and threonine. It is based on the strong acid-induced N-acyl→O-acyl transformations studied earlier by Bergmann, Brand, and Weinmann (2), Desnuelle and Casal (5), and Desnuelle and Bonjour (6), and is briefly outlined in the equations below:



This N-acyl→O-acyl migration might also occur where the ε-amino groups of δ hydroxylysine (26) are linked in a peptide bond.

The treatment of silk fibroin with 97.5% sulphuric acid for three days at 20°C. (8) caused rearrangement in only 66% of the serine residues, the major N-terminal residue detected by the dinitrofluorobenzene method (24) being serine. The non-reactivity of the other serine residues was thought to result

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from factors like steric hindrance. With lysozyme (9) nearly all the serine groups and a third of the threonine residues participated in the reaction. The non-dialyzability of the lysozyme derivative and the absence of the Ehrlich test for tryptophan suggested that cross linking occurred through those residues. Wiseblatt and McConnell (37), who applied the method to wheat gluten, also observed the preferential release of serine amino groups and suggested that the product was highly cross linked.

In the present study the action of anhydrous sulphuric acid in effecting bond transposition in gliadin has been studied from the quantitative aspects of time of reaction and temperature, to determine suitable conditions for further degradation studies on wheat proteins. The gliadin fraction was chosen because of its discrete characteristics. The reactivity of the liberated amino groups towards nitrous acid was determined. This treatment would convert the N-terminal serine residues to glyceric acid. After saponification of the O-acyl bond, the reactivity of the terminal dihydric alcohol towards periodic acid was studied. The above alterations in Elliott's scheme avoid the necessity for protecting the free amino N with a substituent often difficult to remove without other alterations to the material.

An effort has also been made to detect changes in some of the amino acids, arising from sulphuric acid treatment of the protein. Uchino (33) reported that silk fibroin was stable towards sulphuric acid at 7–8°C. for 11 days, but that later there was slow liberation of amino groups. Reitz *et al.* (28) noted that although appreciable hydrolysis of γ-globulin occurred in one week at room temperature there was only 2–3% hydrolysis in 10 days at 4°C. Work on wool (11) indicated considerable non-ionic combination of sulphate ions with the amino groups to form sulphamates. With other proteins (28) it seemed possible to account for all bound sulphate on the basis of ester formation with aliphatic hydroxyl groups, formation of sulphonic acids, and formation of thiosulphates by reaction with the sulphydryl groups. Although some workers observe very little destruction of tryptophan, cystine, and serine (28) other reports (20) indicate appreciable alteration besides the sulphamate formation. When wool was treated with sulphuric acid at low temperatures 10% of the serine and 25% of the arginine was lost. Phenylalanine and tyrosine showed a decrease which, as with tryptophan, may be partly accounted for by sulphonation of the benzene ring.

EXPERIMENTAL

Analytical Methods

Total N was determined by the micro-Kjeldahl method with mercuric oxide catalyst. *Free amino groups* were estimated by the Van Slyke nitrous acid method and a ninhydrin colorimetric procedure (31).

The difference in free amino groups before and after treatment of the materials with pH 9.1 borate buffer for 12–16 hr. was taken as a measure of the *bond transposition* (8).

Standard methods were employed for determination of the following: *amide N* (24), *cystine* (16), *methionine* (13), *arginine* (21), *tryptophan* (10). *Tyrosine* was determined by the 1,2-nitrosonaphthol reaction (25, 34).

Total S was estimated by a combined micro-combustion and volumetric method (32) and *formaldehyde* by the chromotropic acid color reaction (23). The method of Rice, Keller, and Kirschner (30) was used for the identification of the aldehydes as the 2,4-dinitrophenylhydrazones.

Treatment of Gliadin with Sulphuric Acid

One gram of gliadin (N—17.64%, prepared by the method of Blish and Sandstedt (3)) and 10 ml. anhydrous sulphuric acid (prepared by addition of the calculated amount of oleum to 97.5% sulphuric acid) were shaken together for the required time at the temperature desired. The reaction mixture was then poured with stirring into 20 times its volume of cold ether and the precipitated material washed repeatedly with ether. The material was dissolved in the minimum volume of 60% (v/v) ethanol, transferred to a Visking cellophane bag, and dialyzed exhaustively against distilled water. The contents of the bag were lyophilized. The recovery of the products was close to 97% based on nitrogen analyses.

Degradation of the Sulphuric Acid Treated Gliadin

The material obtained above was *acetylated* at pH 5.0 using acetic anhydride (8) or *formylated* with a mixture of formic acid and acetic anhydride (35). *Deacylation* was effected by means of anhydrous MeOH – HCl (1.5 *M* in regard to HCl) under conditions used by Boissonnas and Preitner (4). *Saponification* of the O-acyl bond was achieved using 0.01 *N* NaOH or 0.02 *N* $Ba(OH)_2$ (8).

Deamination of Sulphuric Acid Treated Gliadin

Two methods were employed. In the first, the reaction mixture was treated at 0.5°C. with solid $NaNO_2$ (1 gm. for every gram of protein), added in small portions during 20 min. In the other method 0.5 gm. of the material in 30 ml. 50% acetic acid was treated at room temperature with 1 gm. $NaNO_2$ added in small portions during 20 min. with agitation. The reaction mixtures were exhaustively dialyzed against distilled water and lyophilized. The latter method gave products free of color.

Reaction of Terminal Amino Groups with 1-Fluoro-2,4-dinitrobenzene (DNFB)

Conditions for the reaction and hydrolysis of the products for the release of the dinitrophenyl amino acids were those recommended by Porter (24). Separation of the 2,4-dinitrophenol and 2,4-dinitroaniline from the amino acid derivatives was effected on a chloroform – silicic acid column (19). The dinitrophenyl amino acids were identified by paper chromatography (22) and determined quantitatively by spectrophotometry at 360 mμ (24).

RESULTS

To establish a basis for assessing the results the serine content of gliadin was determined (27) and found to be 4.9% by weight. Gliadin, of molecular weight 30,000, on cleavage of all peptide bonds attached to serine N should therefore yield material containing about 15 fragments with an average chain length of 15–16 residues and with 0.65% amino N (3.7% of total N)

due to exposed serine amino groups. The original gliadin contained 1.01% of the total N as free amino N.

Figs. 1, 2, and 3 contain results obtained for total amino N, amino N arising from bond transposition alone, and amino N other than that arising from bond transposition when samples of gliadin were treated with H_2SO_4 at

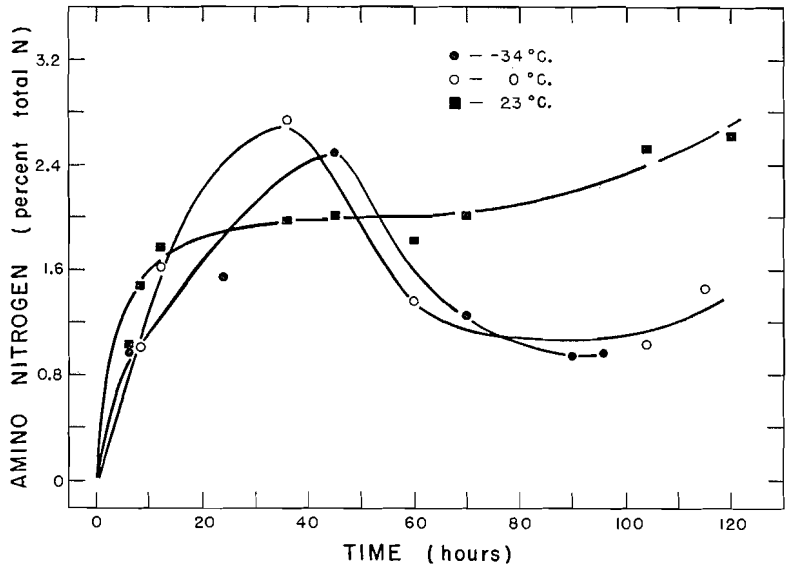


FIG. 1. Effect of time and temperature on free amino N released from gliadin by sulphuric acid.

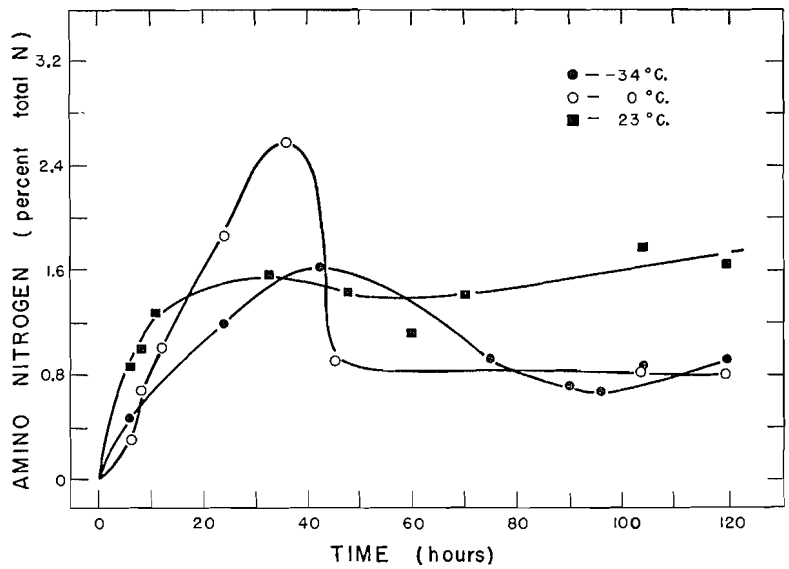


FIG. 2. Effect of time and temperature on the release of amino nitrogen by bond transposition alone during the sulphuric acid treatment of gliadin. Free amino nitrogen expected from a quantitative bond transposition at serine is 3.7% of total nitrogen.

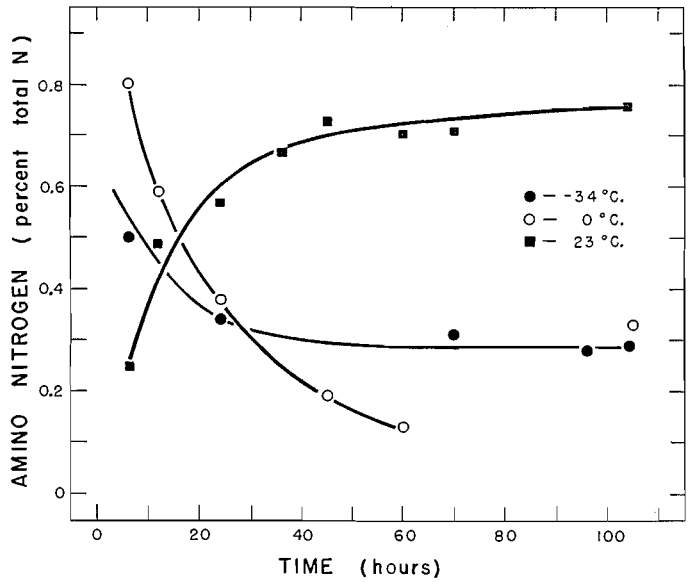


FIG. 3. Effect of time and temperature on amino nitrogen not arising from bond transposition during the sulphuric acid treatment of gliadin.

0°, 23°, and −34°C. for different lengths of time. The data were obtained on lyophilized products, isolated from the reaction mixtures, as described above.

The data indicate that in samples treated at 0°C. and −34°C. the total amino N increases during 35–45 hr. and thereafter falls to 50–65% of the maximum, remaining practically constant. At 23°C. amino groups increase rapidly for about 45 hr. after which there is a slow but continuous rise. Changes in amino N arising specifically from bond transposition follow the same pattern (Fig. 2). The pattern of changes in content of amino N other than those involved in the bond transposition is depicted in Fig. 3. In samples treated at −34° and 0°C. the values decrease gradually to a low value. Since the free amino N content of the original gliadin was measured to be 1.01% the initial decrease at 23°C. is relatively rapid but thereafter there is a progressive increase in free amino groups which may be the result of non-specific hydrolysis.

The difference between analytical values on duplicate preparations was of the order of 10%. The observed trends are therefore significant. The average deviation of the mean for determinations of amino N by the colorimetric method was ±1.5%.

Acylation and Saponification of the Treated Gliadin to Peptides

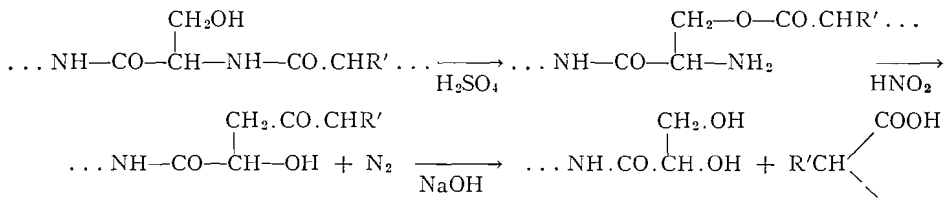
Two samples, one treated at 0°C. for 45 hr. and another treated at 23°C. for 60 hr. with sulphuric acid (0.312% and 0.221% of amino N, respectively, exposed by the bond shift), were subjected to acetylation, saponification, and deacetylation by methods referred to earlier. Material from saponification (after precipitation of barium either by passing in CO_2 at pH 6.5–7.0 or by

adding dilute sulphuric acid) had a high ash content which was retained even after the treatment with MeOH – HCl. To determine the serine end groups, the peptide samples obtained were oxidized with periodic acid. The amounts of formaldehyde obtained corresponded to 107.8 and 92.2% of the theoretical calculated from the amino N values for bond transposition.

The peptide samples were subjected to paper electrophoresis (Whatman 3 mm. 11×30 cm. sheet) using a pH 6.0 phthalate (0.05 *M*) buffer and 0.05 *N* acetic acid. The material was spotted along a line drawn across the middle of the sheet, which had been previously wetted with the electrolyte, and a potential of 220 v. was applied for a period of from two and one-half to three hours. No appreciable part of the material migrated toward either electrode so that although analyses indicated cleavage of the molecule at five to eight sites, no heterogeneity in the resulting material was demonstrable by paper electrophoresis. This observation is similar to results obtained in studies on gluten (37), where the suggestion was made that the material resulting from the treatments employed may consist of cross-linked polypeptide material of colloidal dimensions exhibiting marked homogeneity by physiochemical criteria. It was emphasized that the electrophoretic homogeneity may have resulted from polar groups introduced by the chemical treatments used. It thus appeared of some interest to avoid, at least, the steps of acylation and deacylation.

Deamination with Nitrous Acid and Saponification of the O-Peptide Bond

Some experiments were done therefore on deamination of the products from the sulphuric acid treatment. The reaction should convert the free amino groups to hydroxy groups and subsequent saponification should then yield a mixture of peptides with terminal glyceric acid. The reaction could be represented as follows:



Gliadin treated with sulphuric acid at 0°C. for 45 hr. was deaminated and saponified according to the above scheme of reactions. The isolated product was then oxidized with periodate (37) to determine terminal dihydric alcohols. Formaldehyde* equivalent to 105.5% of that calculated from bond transposition was recovered. The second method of deamination was applied to gliadin treated with sulphuric acid at 23°C. for 60 hr. The sample had a serine amino N value of 0.221% and total amino N 0.298%. After deamination the product had only 0.013% amino N, indicating destruction of 95.6% of the free amino

**Although the amounts of formaldehyde estimated in several samples examined were nearly equivalent to the bond transposition effected, the periodate consumed during the oxidation of the products was found to be several times higher than that expected. The sites in the protein-derivatives responsible for this reduction of periodate have not yet been determined.*

groups. Saponification with NaOH resulted in a product which on periodate oxidation yielded only a trace of periodate ammonia, indicating complete destruction of the serine amino groups. The formaldehyde produced during this oxidation corresponded to 112.8% of the calculated amount. The absence of acetaldehyde in the reaction mixture by the *p*-hydroxydiphenyl test indicated that no threonine residues were involved in the arrangement. This was confirmed when the aldehydes present were reacted with 2,4-dinitrophenylhydrazine and the hydrazones extracted and identified on paper chromatograms. Only one spot corresponding to formaldehyde was present.

The nitrous acid treated samples were hydrolyzed with 6 *N* HCl for 20 hr. as was a control sample of gliadin. The hydrolyzates were chromatographed in two dimensions (36) and the combined intensity of the ninhydrin spots due to serine and glycine was determined spectrophotometrically after extraction with 10% isopropanol. The values indicated that 80–90% of the serine determined as involved in the rearrangement had been destroyed by nitrous acid. However, application of both the dye test (17) and the biuret test (14) to electrophorograms of the two samples (deaminated H_2SO_4-treated gliadin, one at 0°C. for 45 hr. and the other at 23°C. for 60 hr.), prepared as described above, showed a single major component, which moved slowly towards the anode, but did not separate into differing fragments. Both the materials moved a distance of 2.3 cm. in three hours in 0.05 *N* acetic acid.

C-Terminal Groups of Degradation Products

Reaction of the degradation products with carboxypeptidase in a qualitative study indicated the following amino acids occupying C-terminal positions—tyrosine, "leucine",* valine, alanine, glutamic acid, and glycine. No amino acids were released when undegraded gliadin was treated with the enzyme, although application of the modified Schlack and Kumpf procedure (1) for C-terminal groups had indicated the presence of glutamic acid and "leucine" in less than molar amounts (unpublished results of the authors). It is thus possible that bond fission occurred at sites where serine is linked to the above amino acids.

Reaction of Exposed Amino Groups with DNFB

Gliadin was treated with sulphuric acid at 23°C. for four days, precipitated, acetylated, saponified, and then deacetylated with MeOH – HCl and the material fractionated roughly into MeOH – HCl soluble and insoluble fractions (37). The methanol insoluble sample (the amount of material recovered thus accounted for 25% of the nitrogen in the starting material) had very low amino N (0.137%) whereas the soluble fraction had a value of 0.490%. The free serine amino N was 0.082 and 0.183% respectively. The latter sample had a very high ash content and the formaldehyde values on periodate oxidation were abnormally high. It was considered desirable to determine the amount of free serine amino groups by condensing with DNFB and measuring the DNP-serine formed. The dinitrophenyl derivative was hydrolyzed and the DNP-amino acids in the ether extract and aqueous phase examined for their

**No differentiation has been made between leucine and isoleucine.*

identity by paper chromatography (22) after initial purification on silicic acid (19). DNP-serine was found in the ether phase and the aqueous phase contained DNP-histidine. The presence of DNP-histidine is to be expected since histidine occupies the N-terminal position in gliadin (15, unpublished data of the authors). The DNP-serine amounted to 10.65 μM. while the amount theoretically expected from the other analyses was 12.59 μM. Although the recovery for serine amino groups thus appeared to be nearly quantitative, the condensation with the other free amino groups, known to be present from ninhydrin amino N analysis, could not be detected.

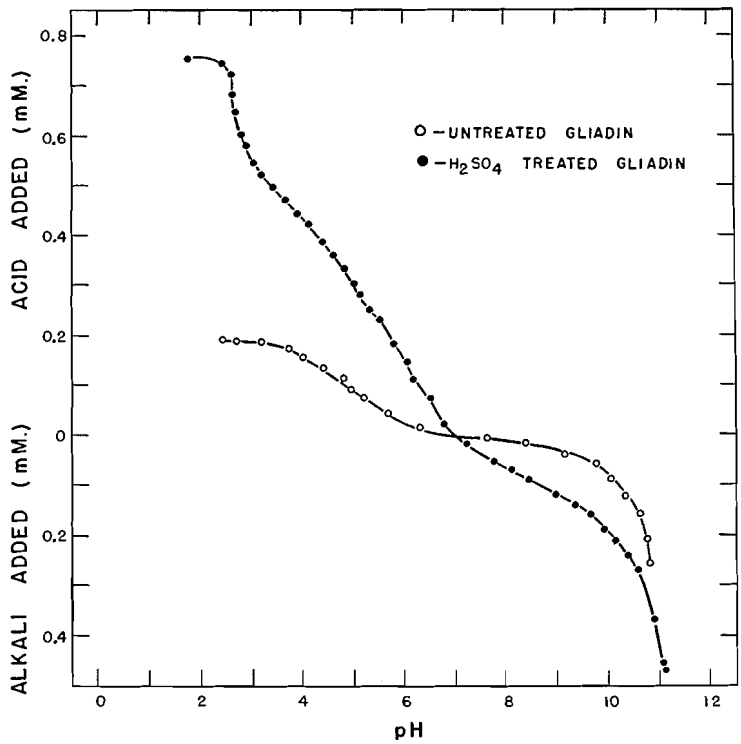


Fig. 4. Titration curve of gliadin before and after treatment with sulphuric acid.

The Effect of Sulphuric Acid Treatment on Some Amino Acid Residues

The titration curves of a sample of gliadin in 60% ethanol, before and after treatment with sulphuric acid at 23°C. for 70 hr., are indicated in Fig. 4. The shape of the curves suggests appreciable changes in the groups titrated in the pH region 2.5–8.5.

Table I contains the results of analyses for bound sulphate, amide N, arginine, methionine, and tyrosine. A single determination of tryptophan in the sample treated for 70 hr. at 23°C. indicated a decrease from 1.15 to 1.02%, of cystine from 2.35 to 1.85%, and of phenylalanine (12) from 5.45 to 4.72% on moisture and ash-free protein.

TABLE I

AMINO ACID CONTENT OF THE SULPHURIC ACID TREATED SAMPLES*

Reaction time, hr.	Reaction temp., °C.	Total S, %	Amide N, as % total N	Methionine	Arginine	Tyrosine
Untreated		0.943	25.6	1.53	4.52	3.5
6	23		23.3	1.11	3.83	2.49
24	23	2.32	23.91		3.67	1.97
70	23		19.75	0.971	3.84	0.71
120	23	3.24	18.69	0.71	3.40	0.65
6	0	1.62		0.75	3.57	2.27
60	0	2.17	25.63	0.67	3.62	0.95
104	0		22.92	0.55	3.41	
6	−34	1.05	20.74	1.22	3.66	2.94
90	−34	1.30	16.20	1.10	3.47	2.14
192	−34	2.40		0.98	2.79	2.29

**All values for amino acids as gm./100 gm. protein.*

DISCUSSION

The results presented show that time of treatment is a factor of importance in the sulphuric-acid-induced acyl migration of peptide bonds in gliadin. Although this study of transposition as a function of time indicates the best conditions (40 hr. at 0°C.), the complete migration of peptide bonds was not obtained under any of the conditions. The shape of the curves in Fig. 3 suggests partial disappearance of amino N during acid treatment at the two lower temperatures, whereas at 23°C. a non-specific hydrolytic cleavage appears to be superimposed. Amino groups might undergo reaction to form sulphamates (11, 29) or be involved in other reactions. If some of the newly formed amino groups participate in such reactions under the conditions of treatment used, it would be impossible to detect or make use of a complete shift of the peptide bonds. Apparent incompleteness in the shift cannot be explained solely on the basis of factors like reactivity of the residues in the protein molecule. Elliott (9) suggested the occurrence of polymerization reactions involving tryptophan residues in the case of lysozyme, but quantitative fission at serine groups and at a third of the threonine residues had occurred. In silk fibroin the cleavage at serine residues was of the order of 66% (8). In gliadin the maximum observed is of the same order (Fig. 2). Thus the effective cleavage of these bonds seems to vary with different proteins.

The present observations indicate that problems arising in the blocking of amino groups and removal of the blocking agent later could be avoided by deamination and that, with gliadin, the removal of the amino groups by nitrous acid is quantitative. This treatment would however convert terminal serine residues to glyceric acid. In theory, this simplification in procedure could distinguish between serine residues rearranged in the first step and those encountered later. Samples of treated protein deaminated in this way showed a decrease in content of serine in the hydrolyzate, of the same order as involved in the bond rearrangement. This in effect constitutes direct evidence of a shift of peptide bonds attached to the serine N in gliadin. Desnuelle (see Ref. 9, p. 140) however has encountered difficulty with some proteins in dimethylating or deaminating the amino groups exposed by sulphuric acid.

The results obtained indicate the incorporation of considerable amounts of undetermined forms of sulphur into the protein during the treatment with acid. Extensive chemical modification of the original protein is also shown by the changes in the content of amide bonds, methionine (cf. 18), arginine, tyrosine, cystine, and phenylalanine. The tryptophan reaction was still strong in some of the products examined. Side reactions involving free amino groups in the sulphuric acid medium are indicated by the gradual diminution of non-serine amino N with time. This perhaps involves, amongst other possibilities, sulphamate formation. The differences observed in the titration curves of gliadin, before and after treatment, possibly result from rather extensive alterations.

The incomplete nature of the rearrangement and the chemical modification of several of the constituent amino acids in the protein severely limit the scope and usefulness of sulphuric acid as a reagent for the selective fission of peptide bonds. The results presented herein do nevertheless provide direct evidence for strong acid-induced acyl migration in gliadin and indicate that a fairly specific cleavage of peptide bonds can thereby be obtained. More satisfactory schemes for selective protein hydrolysis will probably be developed in the future but, for the present, degradation based upon the sulphuric-acid-induced bond transposition remains a useful tool in the study of proteins. Improved understanding of the various effects of sulphuric acid on proteins will lead to rational applications of the procedure and facilitate interpretation of the results.

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