amino acids to the same extent. This is not the case; it has been shown above that chloramphenicol and *p*-chlorophenylalanine affect the incorporation of glutamic acid and phenylalanine (under condition 2) to different extents. In the case of *p*-chlorophenylalanine it is possible to inhibit the incorporation of phenylalanine almost completely without producing significant effect on the incorporation of glutamic acid, and the inhibition of protein synthesis correlates with the effect on phenylalanine. It would seem that inhibition of protein synthesis follows the inhibition of incorporation of that amino acid most sensitive to the inhibitor used. Prevention of protein synthesis by abolishing the uptake of phenylalanine has little or no effect on the rate of glutamic acid incorporation under condition 2, and this confirms the previous studies on glutamic acid incorporation (Gale & Folkes, 1953c) in which it was shown that addition of a complete amino acid mixture increased the rate of glutamic acid incorporation but that omission of phenylalanine from that mixture had little effect. It is clear that the rate of incorporation of a labelled amino acid into the protein fraction of a living cell cannot necessarily be taken as a measure of the rate of protein synthesis within that cell.

SUMMARY

1. Incubation of washed suspensions of *Staphylococcus aureus* with glucose and ¹⁴C-labelled phenylalanine results in incorporation of the isotope into the protein fraction of the cells. The rate of incorporation can be markedly increased by the addition of a complete mixture of amino acids to the incubation mixture.

2. The incorporation of phenylalanine, whether in the presence or absence of the amino acid mixture, is competitively inhibited by *p*-chlorophenylalanine, and non-competitively inhibited by chloramphenicol, aureomycin or terramycin.

3. The incorporation of phenylalanine is more sensitive to the action of chloramphenicol, aureomycin or terramycin than is the incorporation of glutamic acid.

4. When a complete mixture of amino acids is present, an inhibitor may affect net protein synthesis, the incorporation of phenylalanine and the incorporation of glutamic acid to different extents. *p*-Chlorophenylalanine completely inhibits net protein synthesis and phenylalanine incorporation while being without significant effect on glutamic acid incorporation.

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The Reaction of Fluorodinitrobenzene with the α - and ϵ -Amino Groups of Collagen

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The technique developed by Sanger (1945) for the determination of free amino groups in proteins has been applied to collagen and procollagen with the object of gaining information about the form and size of the molecule. So far this technique has only been applied to a few of the insoluble proteins: wool keratin, myosin and tropomyosin. In keratin a comparatively large number of terminal amino groups were detected suggesting that the molecule consists of a relatively large number of polypeptide chains (Middlebrook, 1951; Blackburn, 1949). With myosin and tropomyosin, however, Bailey (1951) was unable to detect the presence of any terminal

amino groups and suggested the possibility of a cyclic molecule.

The evidence available on the structure of collagen from X-ray and electron microscope observations has recently been reviewed by Bear (1952). The fibrils are considered to be formed of regularly coiled polypeptide chains arranged roughly parallel to one another, with regions of similar chemical structure matching transversely. Collagen contains no cystine and there is no evidence for any covalent cross-linkages between the chains, suggesting that, in contrast to keratin, the molecule may consist of a single polypeptide chain. Pauling & Corey (1951),

however, have proposed a structure in which the molecule is considered to be formed of three polypeptide chains, each coiled into a helix and joined to one another by hydrogen bonds.

Porter (1948) has found that, with certain albumins and globulins, not all the ϵ -amino groups react with 1-fluoro-2:4-dinitrobenzene (FDNB) unless the protein is first denatured. With a fibrous protein such as collagen any steric factors affecting the reactivity of such groups are likely to be more pronounced and the non-reactivity may also extend to terminal α -amino groups.

The effect of various treatments on the reactivity of the ϵ -amino groups in collagen towards FDNB and on the liberation of α -amino groups has, therefore, also been considered.

A preliminary account of the work described in this paper was given at the Second International Congress of Biochemistry, Paris, 1952.

MATERIALS

Collagen was prepared from the middle layer of ox hide as described by Bowes & Kenten (1948a).

Procollagen was prepared from fresh calf skin as described by Orekhovich, Tustanovskii, Orekhovich & Plotinkova (1948). The calf skin was shaved, cut into small pieces and disintegrated in a large Wiley mill (A. Thomas Co., Philadelphia, U.S.A.). The mill had to be stopped at frequent intervals and the macerated material removed by hand. The macerate (1800 g.) was extracted twice with 10 l. 0.1M sodium phosphate buffer (pH 8.6) to remove albumins and globulins, and then three times with 10 l. 0.12M sodium citrate buffer (pH 3.62). All extractions were carried out at 4°. The procollagen was precipitated from the citrate extracts by the addition of NaCl to a final concentration of 5% (w/v). The procollagen was purified by dissolving in slightly acidified water (pH 4.0), and dialysing against tap water.

Modification of collagen

Before treatment the collagen was cut into small pieces, of area about 1 cm.², or ground in a laboratory-model Wiley mill to pass a 40-mesh/in. sieve.

Heat shrinkage. Pieces of collagen were heated in water at 65–68° for 15 min., cooled and dehydrated with acetone.

Urea treatment. Powdered collagen was immersed in 8M urea for 24 hr. at laboratory temperature.

Solution in formic acid. Powdered collagen (9.0 g.) was suspended in 500 ml. anhydrous formic acid at 40°. The collagen swelled, and after 18 hr. the greater part had dissolved. The solution was filtered with aid of Celite 545 and Hyflo Super-Cel (Johns-Manville Co., London) and the collagen precipitated by neutralization with 40% (w/v) NaOH. During precipitation the solution was cooled in ice and stirred continuously. The fibrous precipitate was separated and washed with 25% (v/v) aqueous ethanol. The anhydrous formic acid was prepared from the A.R. acid by drying over copper sulphate and distilling under reduced pressure (Garner, Saxton & Parker, 1911).

Alkaline treatment. Pieces of collagen about 0.5 cm.² were immersed in a solution of NaOH and Ca(OH)₂ at

pH 13.0 for 14 days at 20° (Bowes & Kenten, 1948b). Such treatment has been found to dissolve about 5% of the collagen, some as large peptides and some as amino acids and small peptides (Bowes & Kenten, 1948b).

Treatment with hyaluronidase. Powdered collagen (5 g.) was treated with half a 1000 unit ampoule of Hyalase (Bengers, Holmes Chapel, Cheshire) dissolved in 150 ml. 0.33M sodium citrate buffer (pH 4.6) for 16 hr. at 37°. Another 5 g. sample of collagen was treated with heat-inactivated Hyalase under the same conditions. Hyalase is prepared from mammalian testes and may, therefore, be expected to act on chondroitin sulphate as well as hyaluronic acid (Humphrey, 1946; Mathews, Roseman & Dorfman, 1951).

After the enzyme treatment, the collagen was washed by decantation with two 100 ml. portions of the citrate buffer for 8 and 16 hr., followed by two 100 ml. portions of distilled water for similar periods. It was then filtered off, dehydrated with acetone and air-dried.

METHODS

Treatment with fluoro-2:4-dinitrobenzene

Amounts of powdered collagen varying from 1 to 10 g. were treated with FDNB as described by Sanger (1945). For example, 10 g. collagen, suspended in 75 ml. sat. aqueous NaHCO₃ and 150 ml. ethanol, were treated with 8 ml. FDNB in a stoppered flask at room temperature. The time of treatment was usually 3 days, but in some cases was extended to 2 or 3 weeks. At the end of the treatment the reaction mixture was decanted off and the collagen thoroughly washed, first with water, then with ethanol and finally with ether. It was then air-dried.

Removal of soluble yellow material, presumably dinitrophenols, etc., by washing with ethanol was very slow, and in later experiments the DNP-protein was Soxhlet-extracted with ethanol for 16 hr. This was found to remove dinitrophenols almost completely.

The dinitrophenyl (DNP) derivatives of gelatin and formic acid-treated collagen were soluble in water and were not readily precipitated by ethanol or acetone. The reaction mixture was neutralized, extracted with ether, and the aqueous layer saturated with (NH₄)₂SO₄. The gummy precipitates formed were dialysed first against tap water, and then against distilled water for 3 days, and dried *in vacuo* at room temperature.

Hydrolysis, separation and identification of DNP-amino acids

Two methods of hydrolysis were used: (1) 12 hr. with boiling 6N-HCl under reflux; (2) 16 hr. in a sealed tube with 10N-HCl at 105°. Both gave essentially similar results. The latter method was preferred and used for the majority of the experiments since it causes less decomposition of DNP-proline and DNP-hydroxyproline. These residues were considered to be possible end groups in collagen. For the separation of α -DNP-amino acids, 0.5–1 g. of the DNP-protein was usually taken for hydrolysis. Up to 5 g. were taken in attempts to detect a terminal residue in collagen. The hydrolysate was extracted with ether and the α -DNP-amino acids separated on buffered silica gel columns similar to those described by Middlebrook (1949, 1951). The ether extract was first run on a column buffered to pH 6.66

with phosphate buffer (54.6 g. $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} + 53.7$ g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}/\text{l.}$) and developed with ether saturated with the same buffer solution; this separated glutamic and aspartic acids, serine, threonine and glycine. The faster-moving DNP-amino acids were then separated on a column buffered with the pH 6.66 phosphate buffer and developed with CHCl_3 containing 3% (v/v) butanol and saturated with the buffer solution. Additional columns, as described by Sanger (1945) and Blackburn (1949), were used for further separations and confirmation.

For the identification and determination of ϵ -DNP-lysine and ϵ -DNP-hydroxylysine smaller amounts (approx. 10–20 mg. of the DNP-protein) were hydrolysed, the hydrolysates were taken to dryness and run on unbuffered silica-gel columns using 66% (v/v) ethyl methyl ketone in ether, or 30% (v/v) *n*-butanol in CHCl_3 (Sanger, 1945). In later experiments Hyflo Super-Cel was substituted for silica gel.

The identity of the bands observed was checked by running with authentic DNP derivatives, by paper chromatography of the DNP-amino acids, and of the free amino acids following hydrolysis with saturated $\text{Ba}(\text{OH})_2$ for 1 hr. at 105° (Mills, 1950).

The most satisfactory system for the development of paper chromatograms of the DNP-amino acids was found to be *n*-amyl alcohol shaken with an equal volume of 2*N* ammonia solution, the organic phase being used for development and the aqueous phase for saturation of the cabinet.

Recovery of DNP-amino acids

The recovery of DNP-glycine, DNP-proline and ϵ -DNP-lysine when added to collagen and hydrolysed in a sealed tube for 16 hr. with 10*N*-HCl at 105° were 50, 20 and 80%, respectively. The values for DNP-lysine and DNP-glycine were approximately the same as those obtained by Porter & Sanger (1948) under the same conditions, and their values were used in making corrections for the decomposition of the remainder of the DNP-amino acids.

After hydrolysis for 64 hr. only 38% of ϵ -DNP-lysine added to collagen could be recovered.

Determination of DNP-amino acids

The α -DNP-amino acids were determined colorimetrically in 1% (w/v) NaHCO_3 solutions and ϵ -DNP-lysine in 0.75*N*-HCl in 25% (v/v) aqueous ethanol.

The amount of DNP-amino acid derived from a given weight of the original collagen was calculated from the weight of the DNP-collagen taken for hydrolysis, corrected for moisture and ash content, for dinitroaniline present, and for DNP groups attached to ϵ -amino groups. DNP groups attached to terminal amino groups were small in number in all cases, and were neglected. The appropriate corrections were made for decomposition of the DNP-amino acids during hydrolysis.

Determination of lysine and free amino nitrogen

Lysine was determined colorimetrically with ninhydrin following chromatographic separation on an ion-exchange resin (Moore & Stein, 1951).

Free amino nitrogen on the powdered DNP-protein was determined by the Van Slyke procedure using a modified reaction chamber as described by Doherty & Ogg (1943).

RESULTS

Reactivity of ϵ -amino groups

Both ϵ -DNP-lysine and ϵ -DNP-hydroxylysine were identified in hydrolysates of DNP-collagen and DNP-procollagen. The amount of ϵ -DNP-lysine obtained from DNP-collagen was only equivalent to 17 m-moles/100 g. protein, representing only about half the lysine content. Prolonging the time of reaction with FDNB to 14 or 21 days did not increase the amounts of ϵ -DNP-lysine found. With procollagen a rather higher proportion of the lysine was recovered as the DNP derivative. ϵ -DNP-hydroxylysine was not determined quantitatively, but the intensity of the yellow band due to this derivative suggested that the amount present was only equivalent to about half the hydroxylysine content of the collagen.

Following the observation of Porter (1948) that the ϵ -amino groups of some soluble proteins do not all react with FDNB unless the protein is first denatured, the possibility that some of the ϵ -amino groups of collagen are also unreactive was investigated. Treatment in 8*M* urea solution, in alkali, heating in water to 68° or dissolving in formic acid and reprecipitating did not increase the amounts of ϵ -DNP-lysine found after reaction with FDNB, and there was some suggestion that heat shrinkage actually caused a slight decrease (Table 1). Even with gelatin there was no increase in the amount of ϵ -DNP-lysine found.

Only small amounts of nitrogen were evolved from all the DNP-proteins by the action of nitrous acid in the usual Van Slyke procedure, and direct determination of lysine in DNP-substituted preparations of collagen and hyaluronidase-treated collagen gave values of only 4 and 3 m-moles/100 g. respectively. These observations suggested that failure to recover all the lysine as the ϵ -DNP derivative was not primarily due to non-reactivity of a proportion of the lysine to FDNB.

The possibility that the remaining lysine was present as α -DNP-lysine, $\alpha\epsilon$ -diDNP-lysine or as resistant DNP-lysine peptides was next considered. No evidence for the presence of these was obtained from the columns, the only unidentified band being a dirtyish yellow one running very slowly on the ether-phosphate buffer column, considered to be a decomposition product of DNP derivatives. Unless a large number of different peptides was formed, relatively large amounts would have to be present to account for 10 m-moles lysine/100 g. protein. Ether-soluble DNP-peptides would, therefore, almost certainly have been detected since, in some instances, the equivalent of 2–3 g. hydrolysed protein was run on the columns. Much smaller amounts were used for the examination of the aqueous extract owing to the large amount of

ϵ -DNP-lysine present, and small amounts of water-soluble DNP-peptides might not have been detected. Increase in the time of hydrolysis in a sealed tube with 10N hydrochloric acid at 105° from 16 to 64 hr. caused only a slight increase in the ϵ -DNP-lysine found (Table 2). Owing to the extensive decomposition of ϵ -DNP-lysine which occurred during this longer time of hydrolysis, and to the large correction factor which had to be applied, this slight increase was not considered significant.

Terminal amino groups

In addition to yellow bands due to dinitrophenols and dinitroaniline (identified by m.p.) and DNP-amino acids, there was a dirty-yellow material which stayed at the top of the ether-phosphate buffer columns, and a pinkish band running fast on all columns. The first slow band was probably due to some decomposition product of DNP-amino acids, while the fast band which was obtained with hydro-

Table 1. *Free amino groups of collagen and modified collagens*

	α -Amino groups		ϵ -DNP-lysine found	
	(m-moles/ 100 g.)	Mol.wt. containing one residue	(m-moles/ 100 g.)	(% of total lysine residues)
Collagen*		None detected	17	55
Urea-treated				
Aspartic acid	0.07	1 400 000	18	59
Heat-shrunk				
Aspartic acid	0.15	700 000	15	48
Glutamic acid	0.03	3 300 000		
Alkali-treated				
Aspartic acid	0.07	1 400 000	17	55
Glutamic acid	0.17	600 000		
Glycine	0.42	250 000		
Phenylalanine	0.04	2 500 000		
Dissolved in formic acid				
Aspartic acid	0.43	250 000	17	55
Glutamic acid	0.15	700 000		
Glycine	0.65	150 000		
Alanine	0.30	350 000		
Treated with hyaluronidase				
Aspartic acid	0.09	1 100 000	17	55
Alanine	0.16	600 000		
Glycine	0.12	800 000		
Threonine	0.03	3 300 000		
Gelatin (commercial)				
Aspartic acid	0.28	350 000	17	55
Glutamic acid	0.12	830 000		
Glycine	0.57	180 000		
Threonine	0.14	710 000		
Procollagen†				
Aspartic acid	0.06	1 650 000	17	68
Alanine	0.04	2 500 000		

* Lysine content of collagen, 31 m-moles/100 g.

† Lysine content of procollagen, 25 m-moles/100 g.

Table 2. *Recovery of ϵ -DNP-lysine from DNP-proteins*

DNP-protein	Time of hydrolysis* (hr.)	m-moles/100 g. DNP-protein			
		ϵ -DNP- lysine†	Lysine‡	Free NH ₂ -N (Van Slyke)	Lysine not accounted for
Collagen	16	17	4	5	10
	16	17	3	—	11
Alkali-treated collagen	16	17	—	—	—
	64	19	—	—	—

* Hydrolysis in a sealed tube with 10N-HCl at 105°.

† Corrected for decomposition. Recovery of ϵ -DNP-lysine added to collagen and hydrolysed under the same conditions was 80% and 38% after 16 and 64 hr. respectively.

‡ By method of Moore & Stein (1951).

lysates of collagen as well as DNP-collagen was considered to be due to the protein itself and not to any reaction product of FDNB. The eluate corresponding to this band had a smell of charred sugar.

Collagen. No α -DNP-amino acids were detected in hydrolysates of collagen treated with FDNB, even when amounts equivalent to 2–3 g. protein were placed on the columns. It was estimated that using these amounts most amino acids would be detected if present as a terminal group to the extent of one residue/mol.wt. 4 or 5 million. Recovery tests indicated that DNP-proline, which is the DNP-amino acid least stable to hydrolysis, would have been detected if proline were present as a terminal residue to the extent of one residue/mol.wt. 1 500 000.

Procollagen. Small amounts of DNP-aspartic acid and DNP-alanine were found in hydrolysates of DNP-procollagen. It is possible that these small amounts arise from extraneous matter and do not represent terminal amino groups.

Modified collagens. Small amounts of DNP-amino acids were detected in the hydrolysates of the DNP derivatives of the modified collagens and gelatin (see Table 1). Owing to the number of correction factors involved and the small amounts concerned, the values given in Table 1 can only be considered to be correct to within $\pm 20\%$ of the true value.

DNP-Aspartic acid was found in hydrolysates of the DNP derivatives of all the modified collagens and gelatin. With urea-treated collagen this was the only DNP-amino acid found, with heat-shrunk collagen very small amounts of DNP-glutamic acid were also found, and with alkali-treated collagen DNP-glutamic acid, glycine and phenylalanine.

Alanine appeared as an end group when the modification of the collagen involved treatment with acid or slightly acid solutions, e.g. solution in formic acid or extraction with a citrate buffer at pH 4.0 as with procollagen.

The collagen dissolved in formic acid and the commercial gelatin behaved similarly, the DNP derivatives were soluble and the DNP-amino acids found were similar, except that DNP-alanine was found in the formic acid-treated collagen and DNP-threonine in gelatin.

After treatment with hyaluronidase, aspartic acid, alanine, glycine and a trace of threonine were found as terminal residues. No free α -amino groups were found in the collagen treated with the heat-inactivated enzyme under the same conditions, and it seems unlikely that the terminal residues found could have arisen from the hyaluronidase itself. It also seems unlikely that the liberation of α -amino groups from such a variety of amino acids can all be due to removal of polysaccharide actually combined with these groups. It is possible that some hydro-

lysis may have taken place during the treatment, breakdown of polysaccharide having increased the susceptibility of the peptide bonds to hydrolysis at 37° . In this connexion Jackson (1952) reports that treatment of tendon collagen with hyaluronidase increases its solubility in dilute acetic acid.

DISCUSSION

In studying the reactions of a fibrous protein such as collagen it is always difficult to assess whether the reaction has gone to completion or not. It was hoped that combination of FDNB with the ϵ -amino groups of lysine could be used as an indication of the course of the reaction. It was found, however, that, although all the ϵ -amino groups are free to react with nitrous acid (Bowes & Kenten, 1948a), ϵ -DNP-lysine equivalent to only about half these groups was recovered from the DNP-proteins. It was at first thought that this was due to non-reactivity of a proportion of the ϵ -amino groups towards FDNB as observed by Porter (1948) and others. However, the presence of only small amounts of free lysine in the hydrolysates of DNP-collagen, and the small amounts of nitrogen evolved by nitrous acid in the Van Slyke apparatus indicated that, while it was possible that a few of the lysine groups were unreactive, this could not be the only explanation of the low recovery of ϵ -DNP-lysine. Out of 31 m-moles lysine/100 g. collagen, approx. 17 were recovered as ϵ -DNP-lysine (corrected for decomposition during hydrolysis) and 3–4 m-moles were found as unchanged lysine, leaving 10–11 m-moles to be accounted for (Table 2). No evidence was obtained of the presence of α -DNP-lysine, $\alpha\epsilon$ -diDNP-lysine or of DNP-lysine peptides in the hydrolysates; increasing the time of hydrolysis did not significantly increase the amount of ϵ -DNP-lysine found and no yellow bands which might be due to these lysine derivatives were observed on any of the silica-gel columns. There remains the possibility that ϵ -DNP-lysine is much less stable to acid when combined in the collagen than when present as the free amino acid. In other proteins studied there has been no indication of such decomposition, reasonable agreement being found between the ϵ -DNP-lysine and the total lysine content of the protein, provided this has been denatured before treatment with FDNB (Sanger, 1952). If extensive decomposition of ϵ -DNP-lysine does occur, therefore, it would appear to be peculiar to collagen. The possibility that this also applies to other DNP-amino acids should be particularly borne in mind when considering the results of end-group determinations on collagen and modified collagen.

It is interesting to speculate on possible reasons for failure to detect terminal α -amino groups in

collagen. First, the molecule may be very large. Assuming that decomposition of DNP-amino acids during the hydrolytic treatment is no greater when in peptide combination than when free, it was calculated that most terminal amino acid residues would have been detected if present to the extent of one residue/mol.wt. 4 or 5 million. Even if more extensive decomposition occurred it should still have been possible to detect any terminal residue, with the possible exception of proline, if present to the extent of one residue/mol.wt. 1 to 2 million. The molecular weight may, therefore, be of the order of 1 to 2 million at least and probably considerably higher. Average molecular weights of up to 250 000 have been reported for gelatin (Pouradier & Venet, 1950), and as some peptide bonds are almost certainly broken during the conversion of collagen into gelatin it is quite possible that the molecular weight of collagen is several times this figure.

The molecule may be cyclic; this does not at first seem to fit in with the conception of a fibrous protein. There is some indication, however, that end groups may be absent in other fibrous proteins, and Bailey (1951), who was unable to detect any end groups in myosin and tropomyosin, has suggested, on the basis of measurements of molecular size (Tsao, Bailey & Adair, 1951), that the tropomyosin molecule is cyclic.

Finally, there is the possibility that the terminal amino groups are inaccessible or masked in some way. The presence of aspartic acid as a terminal residue in collagen which had received such relatively mild treatments as immersion in urea solution or heating to 68° suggests that it may be a terminal residue inaccessible to FDNB in the original collagen. On the other hand, there is evidence that aspartic acid is readily liberated from proteins. Partridge & Davis (1950) and Adair, Partridge & Davis (1951) have shown that aspartic and glutamic acids are preferentially liberated in the partial hydrolysis of a number of proteins with weak acids, and Blackburn (1950*a, b*) finds that this also occurs when wool is partially hydrolysed with dilute hydrochloric acid. The free α -amino groups of aspartic acid may, therefore, arise from hydrolysis of peptide bonds during the treatment. In view of the importance now ascribed to mucopolysaccharides in the formation of collagen, the possibility of masking of terminal groups by association with chondroitin sulphate or other polysaccharide is worth consideration. Unfortunately the results of the experiment with testicular hyaluronidase were inconclusive, as it is uncertain whether all the amino groups liberated by this treatment arose from removal of polysaccharide. It may be significant, however, that two of the terminal residues found, aspartic acid and alanine, were also found in procollagen.

There is no evidence of any co-ordinate links in collagen, and it is fairly generally agreed that collagen owes its stability to a large number of interchain hydrogen bonds. Several considerations favour the conception of a molecule consisting of a single long polypeptide chain, and the absence of detectable terminal amino groups is most readily explained on the basis of a very large molecule or the masking of this group, most probably aspartic acid, by association with mucopolysaccharide or some other group. If aspartic acid is not a terminal residue, then the peptide bond involving this amino acid is very labile.

A number of other end groups, in addition to aspartic acid, were found in the modified collagens; these probably arise from hydrolysis of peptide bonds, suggesting that certain peptide bonds involving the amino groups of glutamic acid, glycine, alanine and to a lesser extent threonine and phenylalanine are particularly labile. There is, however, the possibility that some of these DNP-amino acids arise from the uncovering of α -amino groups by the splitting off of DNP-aspartic acid during the treatment with FDNB. This has been found to occur with 'old yellow enzyme' (Weygand & Junk, 1951).

The detection of an appreciable number of free α -amino groups in collagen which has been dissolved in formic acid and reprecipitated calls for some comment. It is often assumed that collagen can be recovered unchanged from formic acid solution, but the present results suggest that the product obtained more nearly resembles gelatin than the original collagen, the number of free α -amino groups actually exceeding those of a commercial gelatin.

SUMMARY

1. The reaction of fluorodinitrobenzene with the amino groups of collagen, modified collagen and procollagen has been studied.

2. Only 55–60 % of the lysine could be recovered as the ϵ -dinitrophenyl (DNP) derivative from collagen, modified collagen or gelatin which had been treated with fluorodinitrobenzene. Only small amounts of free amino nitrogen and unchanged lysine were found in the DNP proteins leaving approx. 30 % of the lysine unaccounted for. The possibility of decomposition of ϵ -DNP-lysine during hydrolysis, and of the presence of acid-resistant ϵ -DNP-lysine peptides is considered.

3. No terminal residues were detected in collagen, and possible reasons for this are discussed.

4. Small amounts of various end groups were found in procollagen, the modified collagens, and gelatin. Aspartic acid was most frequently found as a terminal residue, followed by glutamic acid,

glycine, alanine and threonine. With the possible exception of aspartic acid these are considered to arise from hydrolysis of peptide bonds during the treatments. Aspartic acid is either a terminal residue which is inaccessible to fluorodinitrobenzene

or the peptide bond involving its α -amino group is especially labile.

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The Catalytic Effect of Molybdate on the Hydrolysis of Organic Phosphates

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Lutwak & Sacks (1953) recently investigated the effect of molybdate on the hydrolysis of organic phosphates at a temperature of 100°. Two series of experiments were carried out, one at a pH of about 5.5 and the other in *N* sulphuric acid. The inorganic phosphate formed after certain time intervals was determined by two methods: (a) by direct colorimetric estimation according to Fiske & Subbarow (1925), and (b) by precipitation with magnesia mixture, followed by a colorimetric analysis of the dissolved precipitate. Both methods were in agreement when samples were analysed, which did not contain molybdate during heating; on the other hand, when organic phosphates were heated together with molybdate, the inorganic phosphate estimated by the direct colorimetric method was considerably in excess of that estimated after precipitation with magnesia mixture; it was, moreover, often in excess of the theoretical value for complete hydrolysis, in one case by about 400%. These

results were interpreted as indicating the formation of organophosphomolybdate complexes, not precipitable by magnesia mixture, but reducible by the Fiske-Subbarow reagent. If this interpretation were correct, it would cast doubt on the significance of some of the results obtained earlier by Weil-Malherbe & Green (1952) in an investigation in which the molybdate effect was studied under different experimental conditions and with a different analytical technique. It was therefore of interest to find out (a) whether the effect of Lutwak & Sacks would be observed with their analytical techniques under the milder experimental conditions that were used in most of the experiments of Weil-Malherbe & Green, i.e. at a temperature of 80° or less and in the pH range between 3 and 6; and (b) whether it could be observed under the more rigorous conditions of Lutwak & Sacks with the analytical technique of Weil-Malherbe & Green who used a stannous chloride method of phosphate