The Effect of Hydrolysis of Proteins on their Ultraviolet-Absorption Spectra near 210 m μ

BY M. P. TOMBS AND N. F. MACLAGAN

Department of Chemical Pathology, Westminster Medical School, London, S.W. 1

(Received 5 December 1961)

Proteins show a region of intense absorption at wavelengths shorter than 230 m μ that was first extensively investigated by Goldfarb, Saidel & Mosovitch (1951), who concluded that a large part of the absorption was due to the peptide bond. The only other major contribution came from the aromatic amino acid residues.

Since proteins generally have similar peptide-bond contents, it is to be expected that near 210 m μ they will all have similar specific absorptions, provided that the aromatic amino acid content is not unusually high or low (Tombs, Cooke, Souter & MacLagan, 1960). Use has been made of this similarity in methods for the assay of mixtures of proteins, at either 215 and 225 m μ (Waddell, 1956; Bendixen, 1957; Murphy & Kies, 1960) or 210 m μ (Tombs, Souter & MacLagan, 1959). The specific absorption of proteins at 210 m μ is approximately 200, and this great sensitivity is useful, e.g. for analysis of the effluent in protein chromatography, since as little as 1 mg. can be satisfactorily fractionated (Tombs, Cooke, Burston & MacLagan, 1961).

Since during proteolysis peptide bonds are split, a decrease in the absorption at 210 m μ is to be expected, and it might be possible to devise a very sensitive procedure for the determination of the number of bonds split. This approach has been successful in following the splitting of acetyl-methionine at 238 m μ (Mitz & Schlueter, 1958) and of simple peptides near 230 m μ (Schmitt & Siebert, 1961). On the other hand, the action of pepsin has been reported to produce a small rise in the absorption at 210 m μ (W. H. Taylor, personal communication). This is at first sight contrary to expectation and requires some explanation. We have therefore investigated the change in ultraviolet absorption produced by acid and enzymic hydrolysis, in an attempt to reconcile the slight rise reported in peptide hydrolysis with the decrease expected as a result of peptide-bond splitting.

METHODS AND MATERIALS

Proteins. Crystalline human and bovine albumin, protamine sulphate (salmine), bovine γ -globulin, ovalbumin and edestin were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and were used without

further purification. Crystalline trypsin, chymotrypsin and pepsin were obtained from the same source.

Ultraviolet-absorption measurements. These were made on a Hilger Uvispek spectrophotometer H 700 fitted with a synthetic silica prism. In previous work (Tombs *et al.* 1959) some difficulty was encountered in measuring at 210 m μ owing to insufficient energy levels passing the optical system. The use of synthetic prisms eliminates most of the difficulties previously described and no more than ordinary precautions were necessary to obtain satisfactory measurements. Matched cuvettes of 1 cm. or 0.5 cm. path length were used, with water as a reference standard throughout.

Acid hydrolysis. Solutions (1%) of protamine sulphate, bovine and human albumin and γ -globulin were mixed with an equal volume of fresh conc. HCl (A.R.). The resulting solution contained 0.5% of protein and was approximately 6N in HCl. Duplicate portions of 0.3 ml. were withdrawn, diluted to 50 ml. with water and the absorption at 210 m μ was determined. The mixtures were then heated at 98–100° in a boiling-water bath and similar samples removed at intervals.

Enzymic hydrolysis. Pepsin was used in 0.06N-HCl (final pH 1.4–1.7); trypsin and chymotrypsin were used in phosphate buffer, pH 7.79 (0.780 g. of NaH₂PO₄·2H₂O and 16.117 g. of Na₂HPO₄·12H₂O/l.). The temperature was 37° in all cases. Enzymes were dissolved in the appropriate solvent and allowed to stand until no turbidity could be detected by eye. Substrate solutions were also examined for absence of turbidity and where necessary centrifuged to clear.

Substrate concentration in the digestion mixture was 5 or 10 mg./ml. For determination of changes in u.v. absorption duplicate samples (0.3 ml.) were removed and diluted to 50 ml. with water. Extinctions remained constant for several hours after dilution; duplicates agreed within 3% and the estimated precision of measurement was $\pm 1\%$.

Two methods were used to follow proteolysis: the appearance of material soluble in 3.75% (w/v) trichloroacetic acid and the direct formol titration (Taylor, 1957). For the former method samples (1 ml.) were added to 2.5 ml. of 5% trichloroacetic acid, allowed to flocculate, centrifuged and the absorption of the supernatants at 280 m μ was determined. For formol titrations samples (3 ml.) were mixed with 10 ml. of 10% (w/v) formaldehyde solution previously adjusted with 0.1N-NaOH to pH 8.9, and then titrated to pH 8.9 with 19.4 mN-NaOH. Initial reaction mixtures were taken as blanks; the increment in titration then measured released carboxyl groups. From the appropriate mean residue weights of the substrates (Tristram, 1953) it was possible to calculate the titration expected as a result of complete hydrolysis and to express

results as a proportion of the total number of peptide bonds split. The pepsin results were less precise than others because of the relatively large blank titrations. Even so, the estimated maximum error was $\pm 1\%$ of the total number of peptide bonds.

Trichloroacetic acid-solubility measurements were used mainly in preliminary experiments: they have the advantage of sensitivity but do not give a direct measure of the extent of peptide-bond splitting and their usefulness ends when all the digest is soluble. Comparison of formal titrations and trichloroacetic acid solubility showed that, for peptic digestion of ovalbumin and tryptic and chymotryptic digestion of bovine serum albumin, there was a linear relationship between the percentage of bonds split and solubility in trichloroacetic acid.

In the former case ovalbumin was rendered 70% soluble at 12% bond fission and in the latter bovine albumin became 50% soluble at 10% bond fission. In contrast, pepsin rendered edestin almost completely soluble at 7% and albumin at 5% bond fission.

Fig. 1. Effect of 6N-HCl on the extinction at 210 $m\mu$ of $\circ$, bovine albumin (upper curve), protamine sulphate (lower curve), $\bullet$, human albumin, $\bullet$ bovine γ -globulin. Heating at 100° was commenced at zero time: the variations shown to the left of zero are the effect of acidification at room temperature. All results have been calculated to 1% of protein; measurements were on approximately 0.01% solutions.

RESULTS

Acid hydrolysis. Fig. 1 shows the effect of acidification and subsequent heating on albumin, γ -globulin and protamine. Acidification at room temperature caused an immediate increase of the absorption of albumin at 210 $m\mu$, which then remained constant until heating was commenced. After heating, the absorption dropped rapidly for all the proteins, reaching a fairly constant low amount after 10 hr. The increase on acidifying and the subsequent drop on heating were: for bovine albumin, 11 and 59%; for human albumin, 10 and 46%; for bovine γ -globulin, 3 and 48%; for protamine, 3 and 62%. Unacidified control solutions of the proteins showed a negligible increase in absorption (1–2%) after 24 hr.

Fig. 2 shows the absorption spectra from 205 to 240 $m\mu$ of intact albumin and protamine, their hydrolysates and solutions of the aromatic amino acids made up in 0.12N-HCl to correspond to the intact protein solutions in composition. (Human albumin: phenylalanine, 7.8%, tyrosine, 4.7%,

Fig. 2. Absorption spectra: (1a) intact human albumin in water; (1b) acid hydrolysate of human albumin in 0.12N-HCl; (1c) aromatic amino acids equivalent to human albumin in 0.12N-HCl; (2a) intact protamine sulphate; (2b) hydrolysate of protamine sulphate. All results have been calculated to 1% of protein or its equivalent in hydrolysate or aromatic amino acids. Measurements were made on 0.01% protein solutions or their equivalents.

tryptophan, 0.2%. Protamine: nil. Tristram, 1953.) It will be seen from Fig. 2 that the decrease in absorption produced by hydrolysis extends down to $235\text{ m}\mu$ and that the aromatic amino acids account for most of the residual absorption. Typical findings are shown for human albumin and protamine; the results with bovine albumin and γ -globulin were similar to those of the human albumin.

The conditions employed for acid hydrolysis are known to split most of the peptide bonds in the molecule (Hirs, Stein & Moore, 1954) and the decrease in absorption shown in Figs. 1 and 2 must have been due to fission of peptide bonds. There remained a possibility that a part of the decrease might have been due to destruction of the aromatic amino acids, such as tryptophan, which is known to be destroyed on acid hydrolysis (Desnuelle, 1953). To test this possibility a mixture of the aromatic amino acids in proportions corresponding to those present in bovine γ -globulin and albumin were heated in acid solution under the same conditions as used for hydrolysis. The γ -globulin amino acid mixture dropped about 5% and the albumin mixture actually rose 10% in absorption in 24 hr. Although conditions used can only approximate to those obtaining during hydrolysis of protein, the results make it unlikely that the drop in absorption during acid hydrolysis could have been due to destruction of aromatic amino acids. This possibility can certainly be excluded for protamine, which contains no such acids: its main constituent, arginine, is not appreciably destroyed by acid hydrolysis (Hirs *et al.* 1954).

Enzymic hydrolysis. The effect of enzymes on u.v. absorption was in general less dramatic than that of acid because of the smaller degree of hydrolysis produced. The results of enzymic hydrolysis depended upon the nature of the substrate and the extent of peptide-bond fission. Fig. 3 illustrates the effect of trypsin on protamine: approximately 15% of the u.v. absorption was lost when 16% of the peptide bonds were split. Similar results were obtained with higher enzyme:substrate ratios. The loss of u.v. absorption followed the increase in bond fission closely, and no sign of a rise in absorption or delay in falling was observed. The magnitude of the decrease was roughly that expected in view of the results of acid hydrolysis. With more complex substrates rises in absorption, or at least delayed decreases, were seen. This is shown in Fig. 4, where tryptic hydrolysis caused fission of 5% of the peptide bonds. This would have been expected to produce a decrease of 2–3% in the absorption; instead it remained constant. With a higher concentration of trypsin a small increase was found. Chymotrypsin split a further 5% of the peptide bonds, causing a further small

Fig. 3. Tryptic hydrolysis of protamine at 37° ; 0.2 mg. of trypsin/ml. and 5 mg. of protamine/ml. (a) Change in E at $210\text{ m}\mu$; (b) extent of peptide-bond fission as shown by formol titration. For further details see text.

Fig. 4. Enzymic hydrolysis of bovine albumin at 37° . The initial enzyme-substrate mixture contained 10 mg. of trypsin and 500 mg. of albumin in 50 ml. of buffer. At point C 10 mg. of chymotrypsin was added; at point P the pH was adjusted to 1.9 with 3 drops of conc. HCl and 10 mg. of pepsin was added. Increases in E at points C and P are due to the added enzyme. Turbidity appeared after the addition of pepsin. The lower curve shows the extent of bond fission, determined by formol titrations.

Fig. 5. Peptic hydrolysis of bovine albumin. (a) Changes in u.v. absorption. The initial mixture contained 10 mg. of albumin and 0.05 mg. of pepsin/ml. At A an equal volume of conc. HCl was added and the mixture heated at 100°. These absorptions have been multiplied by 2 to compensate for the dilution involved. Note the change in the scale of the abscissa at 0.60. (b) Extent of bond fission, determined by formol titration.

increase, but when about 10 % of the bonds had been split a decrease of about 3 % of the initial u.v. absorption occurred. Pepsin split still more peptide bonds, but also produced visible turbidity and the increase in absorption was of doubtful significance. This initial increase was not peculiar to albumin, since tryptic or chymotryptic digestion of ovalbumin also produced small increases (1–2 %) in the absorption at 210 $m\mu$. Controls lacking either enzyme or substrate showed no significant variation.

The situation was rather different in peptic digestion. This was of necessity at low pH, and the substrates were denatured. Fig. 5 shows the increase in absorption observed with albumin after fission of approximately 8 % of the peptide bonds. The digest was then acid-hydrolysed, producing a decrease in absorption very similar to that produced by acid alone. Further peptic digestions with a greater amount of enzyme are shown in Fig. 6. In this case albumin shows a slight decrease of 1.5 % in absorption after the fission of about 10 % of the bonds. However, edestin showed a delayed decrease of 3 % after fission of 8 % of the bonds, and ovalbumin a decrease of 4 % after 14 %

Fig. 6. Peptic hydrolysis of O, edestin, ●, ovalbumin and ●, bovine albumin at 37°. (a) Variation in absorption at 210 $m\mu$. Substrate concn. 10 mg./ml. For bovine albumin, 1 mg. of pepsin/ml.; for the remainder, 0.5 mg. of pepsin/ml. (b) Extent of peptide-bond fission determined by formol titration.

bond splitting. These decreases are slightly less than would have been expected from the results of acid hydrolysis.

DISCUSSION

The effects of acid hydrolysis on the u.v. absorption of the proteins studied were in accord with expectation since in most cases an initial rise due to denaturation was followed by a progressive fall of about 50 % of the initial value as hydrolysis proceeded. Previous calculations of the approximate contribution made by the peptide bond to the absorption at 210 $m\mu$ was in the neighbourhood of 60 % (Goldfarb *et al.* 1951), and the absorption of the released amino and carboxyl groups is relatively low so that the results of acid hydrolysis are readily explicable on this basis. Confirmation was also obtained by studying the residual absorption of the hydrolysate, which was similar to that of the aromatic amino acids contained in the original protein. The fact that the initial rise on acidification was associated with denaturation was confirmed by the appearance of the characteristic denaturation difference spectrum at 234 $m\mu$ and at 270–290 $m\mu$ (Glazer & Smith, 1960). Protamine and γ -globulin showed negligible initial increases, the former because of its simple structure and the latter possibly because of denaturation during preparation.

With enzymic action, both falls and rises in u.v. absorption were recorded in different cases. It is generally held that the initial action of proteolytic enzymes involves partial denaturation since fission of even a few bonds may cause extensive unfolding of complex substrates. The initial changes obtained with acid suggest that structural changes of this kind are associated with an increase in u.v. absorption, so that the decrease expected as a result of bond fission will be opposed by an increase. The increase will depend on the nature of the substrate and on the number and kind of bonds split in any particular instance. The situation is clearly complex but may produce two extreme possibilities: a decrease in absorption with no preliminary increase or an increase in absorption with no subsequent decrease. The former is exemplified by protamine, where the result of enzymic hydrolysis was very similar to that of acid. This may be related to the relative simplicity of the molecule. On the other hand, with albumin fission of less than 8% of the bonds failed to cause a decrease in absorption, though further splitting did produce small decreases (Fig. 4); subsequent hydrolysis with acid caused the loss of about 60% of the absorption, as expected (Fig. 5). The failure to decrease on limited fission of the peptide bonds must have been due to an increase in absorption following on structural rearrangement of the molecule. Since 8–10% fission would have been expected to produce a decrease in absorption of 5%, and failed to do so, the opposing increase must have been of the order of 5% of the initial absorption.

An increase of 5% in absorption as a result of structural rearrangement is to be compared with the 10% increase found on acid-denaturation. Too close a parallel cannot be drawn between enzymic and acid-denaturation, however, if only because with enzymes actual fission of the peptide chain occurs, which is absent with acid. This is emphasized by the result of peptic hydrolysis when the substrates were acid-denatured before enzymic action commenced.

Fig. 6 shows that albumin and to a lesser extent edestin still show an increase in absorption opposing the decrease due to fission. Ovalbumin in these circumstances shows a steady decrease but the magnitude was slightly less than expected. It must be concluded that, even if the substrate is already denatured by acid, fission of the peptide chain can still cause structural alterations producing an increase in absorption. Large changes in absorption in this region of the spectrum have been described (Imahori & Tanaka, 1959) as a result of helix-random-coil transformations in synthetic poly-L-glutamate.

It appears from our results that changes in u.v. absorption below 230 m μ could be used to follow the acid hydrolysis of any protein and the enzymic

hydrolysis of relatively simple peptides, but is of little use in following the enzymic hydrolysis of complex proteins.

SUMMARY

1. The effect of acid and enzymic hydrolysis on the ultraviolet-absorption spectra of some typical proteins has been studied in the region below 240 m μ .

2. Cold 6N-hydrochloric acid increased absorption at 210 m μ of bovine and human albumin by 10% but had little effect on that of bovine γ -globulin or protamine sulphate.

3. Heating in 6N-hydrochloric acid produced in all proteins studied a rapid drop in absorption at 210 m μ to approximately half the initial value.

4. Enzymic hydrolysis also tended to produce an initial rise followed by a fall in absorption at 210 m μ . With simple substrates (protamine) no initial rise was seen. With albumin, fission of less than about 8% of the peptide bonds caused a net rise and further fission produced a decrease.

5. Correlation of these results with the extent of peptide-bond fission shows that the fall in ultraviolet absorption on hydrolysis was due to peptide-bond splitting; the initial rise with complex proteins was ascribed to structural changes.

We are grateful to the British Empire Cancer Campaign for financial support and to Mr D. Janikoun and Miss M. Apsey for technical assistance.

REFERENCES

- Bendixen, G. (1957). *Nord. med. Tidskr.* **58**, 1487.
Desnuelle, P. (1953). In *The Proteins*, vol. 1, p. 158. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Glazer, A. N. & Smith, E. L. (1960). *J. biol. Chem.* **235**, P.C. 43.
Goldfarb, A. R., Saidel, L. J. & Mosovitch, E. (1951). *J. biol. Chem.* **193**, 397.
Hirs, C. H. W., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 941.
Imahori, K. & Tanaka, J. (1959). *J. molec. Biol.* **1**, 359.
Mitz, M. A. & Schlueter, R. J. (1958). *Biochim. biophys. Acta*, **27**, 168.
Murphy, J. B. & Kies, M. W. (1960). *Biochim. biophys. Acta*, **45**, 382.
Schmitt, A. & Siebert, G. (1961). *Biochem. Z.* **334**, 96.
Taylor, W. H. (1957). *Analyst*, **82**, 488.
Tombs, M. P., Cooke, K. B., Burston, D. & Maclagan, N. F. (1961). *Biochem. J.* **80**, 284.
Tombs, M. P., Cooke, K. B., Souter, F. & Maclagan, N. F. (1960). *Proc. 7th Colloq. Protides biol. Fluids, Bruges*.
Tombs, M. P., Souter, F. & Maclagan, N. F. (1959). *Biochem. J.* **73**, 167.
Tristram, G. R. (1953). In *The Proteins*, vol. 1, p. 181. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
Waddell, W. J. (1956). *J. Lab. clin. Med.* **48**, 311.