192. *An X-Ray Study of the Hydration and Denaturation of Proteins.**

By W. T. Astbury and R. Lomax.

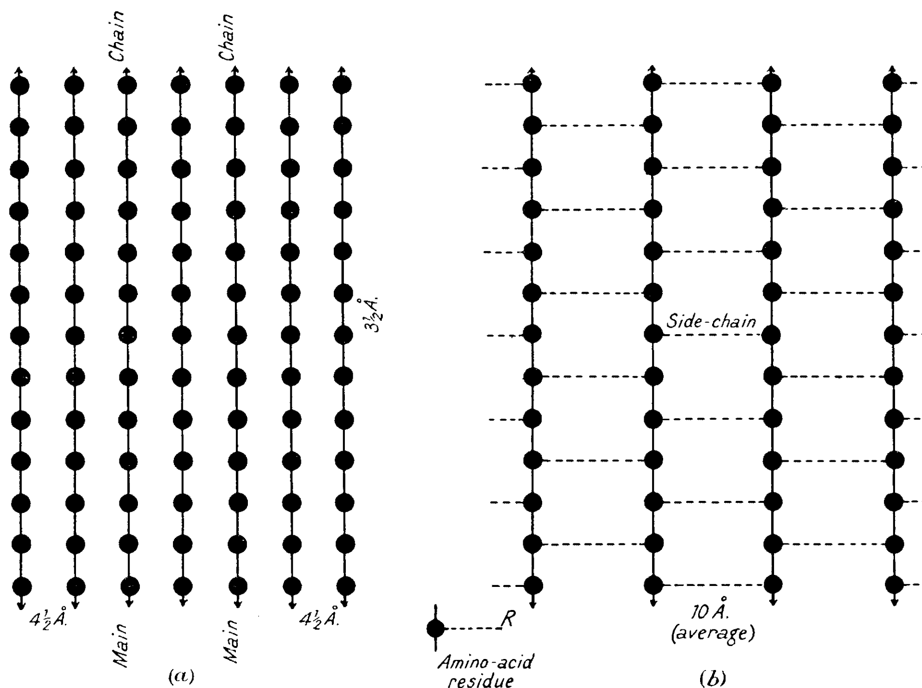
Comparative studies of the *X*-ray photographs given by proteins (Astbury, *Trans. Faraday Soc.*, 1933, **29**, 193; Cold Spring Harbor Symposia on Quantitative Biology, 1934, **2**, 15; *Kolloid-Z.*, 1934, **69**, 340), in particular of the " fibre photographs " given by the keratin of mammalian hairs (Astbury, *Ann. Reports*, 1931, **28**; Astbury and Street, *Phil. Trans.*, 1931, *A*, **230**, 75; Astbury and Woods, *ibid.*, 1933, **232**, 333), lead to the conclusion that a fully extended polypeptide chain is characterised by two principal side-spacings approximately at right angles to each other, one (about $4\frac{1}{2}$ Å.) arising from the effective thickness of the " backbone " of the chain, and the other (about 10 Å.) from the average lateral extension of the side-chains standing out from the amino-acid residues. These

* Based on a contribution to the discussion on " Applications of *X*-Rays and Spectroscopy to the Elucidation of Chemical Structure," held at the University of Manchester, November 9th, 1934 (for abstract, see *J. Soc. Chem. Ind.*, 1934, **53**, 979).

two spacings thus appear to represent the two principal modes of linkage of neighbouring protein chains: they may be illustrated, purely diagrammatically, by Figs. 1*a* and 1*b*.

The identification of the side-chain spacing rests largely on the results of the *X*-ray examination of the interaction of proteins with water, *e.g.*, the action of steam, hot water, or cold dilute caustic alkali on β-keratin (stretched hair), where it can be shown geometrically that the reaction is associated almost exclusively with a disturbance in the longer side-spacing (9·8 Å.). Collagen, gelatin, and related structures show analogous well-marked changes simply on wetting, and in this case there is a continuous increase in the side-chain spacing as the water penetrates between the molecular chains. Figs. 2*a* and 2*b*, respectively, show the action of steam on β-keratin and the action of cold water on elastoidin fibres * from a fin of *Carcharias.* In Fig. 2*a* certain reflexions are drawn out along the hyperbolic "layer-lines," and the distribution of these elongated spots (which present a normal

FIG. 1.



The two principal modes of linkage of neighbouring protein chains: (a) *"backbone" linkage:* (b) *side-chain linkage.*

appearance in *X*-ray photographs of untreated β-keratin) indicates that there has occurred, on steaming, a unidirectional disturbance parallel to the larger side-spacing. Fig. 2*b* is a double photograph taken on one film, the top half being of elastoidin fibres after drying over phosphoric oxide, and the bottom half after wetting with water. It will be seen that the equatorial reflexion nearest the centre (the side-chain reflexion) is displaced and drawn out towards the centre of the photograph by the action of the water.

Most proteins, however, as ordinarily available, do not give clear-cut crystal effects, but only diffuse ring photographs showing little more than what appear to be the backbone and side-chain spacings of polypeptide chains. It is important, therefore, to test this conclusion also by swelling reactions, and to this end a number of non-fibrous proteins were photographed both dry and after adsorption of water or alcohol. The proteins examined were: ordinary egg-white, boiled egg-white, serum albumin, serum albumin

* For these fibres we are indebted to Prof. W. J. Schmidt, of Giessen (see Schmidt, "Polarisationsoptische Analyse des submikroskopischen Baues von Zellen und Geweben," p. 601, in Abderhalden's "Handbuch der biologischen Arbeitsmethoden," 1934).

denatured by heat, pepsin,* trypsin,* zein,† commercial casein, tobacco-seed globulin, squash-seed globulin, and edestin from hemp seed: X-ray photographs of eight of these at ordinary humidity are shown in Figs. 3, (1)—(8). For the purposes of the discussion given below they are arranged in descending order of "crystallinity"; *i.e.*, as we proceed from (1) to (8), the X-ray reflexions become both fewer and more diffuse.

All the photographs were taken with Cu-K_a rays, the protein being packed into a small hole drilled through a microscope slide. The camera was in the form of a closed box in which the specimen could be kept wet or (except just below saturation) at approximately constant humidity at laboratory temperature. In front of the photographic film was a metal slider by means of which first one half of the film, and then the other, could be protected from the diffracted X-rays. In this way, two comparable photographs could be taken on one and the same film without disturbing the adjustment of either specimen or film—the latter, of course, was carefully shut off from the effects of the various humidity changes.

Figs. 4, (1)—(10), show the double photographs so obtained of ten proteins, the left half always being that of the specimen after drying for about 2 days over phosphoric oxide, and the right half after exposure to water or alcohol vapour or actual wetting with water.

Both the ordinary and boiled egg-white were first dried over phosphoric oxide, and then allowed to take up the humidity required. The serum albumin was denatured by gently heating a solution in distilled water till precipitation occurred.

FIG. 2*a*.

X-*Ray fibre photograph of human hair stretched in steam to double its initial length ("steam-set" β-keratin).*

FIG. 2*b*.

Comparable X-*ray fibre photographs of dry (top) and wet (bottom) elastoidin fibres from a fin of* Carcharias.

FIG. 3.

X-*Ray powder photographs of proteins at atmospheric humidity:* (1) *Tobacco-seed globulin,* (2) *squash-seed globulin,* (3) *edestin from hemp seed,* (4) *boiled egg-white,* (5) *pepsin,* (6) *ordinary egg-white,* (7) *casein,* (8) *zein.*

FIG. 4.

X-*Ray powder photographs of proteins after being dried over phosphoric oxide (left), and then exposed to water or alcohol vapour or wetting with water.* (1) *Ordinary egg-white, dry and at* 100% *relative humidity;* (2) *boiled egg-white, dry and at* 100% *relative humidity;* (3) *serum albumin, dry and at* 100% *relative humidity;* (4) *denatured serum albumin, dry and at* 100% *relative humidity;* (5) *trypsin, dry and at* 90% *relative humidity (plus ammonium sulphate);* (6) *zein, dry and wet with water;* (7) *zein, dry and in alcohol vapour;* (8) *squash-seed globulin, dry and wet with water;* (9) *squash-seed globulin, dry and in alcohol vapour;* (10) *pepsin, dry and wet with water (second effect).*

FIG. 5.

X-*Ray powder photographs of normal and denatured albumins at atmospheric humidity:* A_1, *normal egg white;* B_1, *normal serum albumin;* A_2, *boiled egg white;* B_2, *heat-denatured serum albumin.*

DISCUSSION.

Protein Powder Photographs.—Fig. 3 illustrates well the general similarity between X-ray powder photographs of proteins as ordinarily available: in every case the diffraction pattern either consists solely of, or is based on, a pair of rings of roughly constant dimensions, and, as pointed out above, existing evidence suggests strongly that the inner ring is to be associated with the side-chain spacing, and the outer with the backbone spacing, of polypeptide chains.

The table gives the spacings of the various rings shown by the photographs of Fig. 3. It will be apparent that the backbone spacing is much more constant than the side-chain spacing—the former has a mean variation of less than 0·1 Å. about its mean ($4{\cdot}5_9$ Å.), whereas the latter has a mean variation of more than 0·5 Å. about its mean ($10{\cdot}4_2$ Å.). This in itself provides a sound argument in favour of the proposed interpretation of the two spacings, for, although we should indeed expect the average side-chain separation of poly-

* Kindly sent by Prof. J. H. Northrop (see *J. Gen. Physiol.*, 1930, **13**, 739; 1932, **16**, 267).

† Both commercial zein and a preparation by Prof. A. C. Chibnall.

Fig. 3.

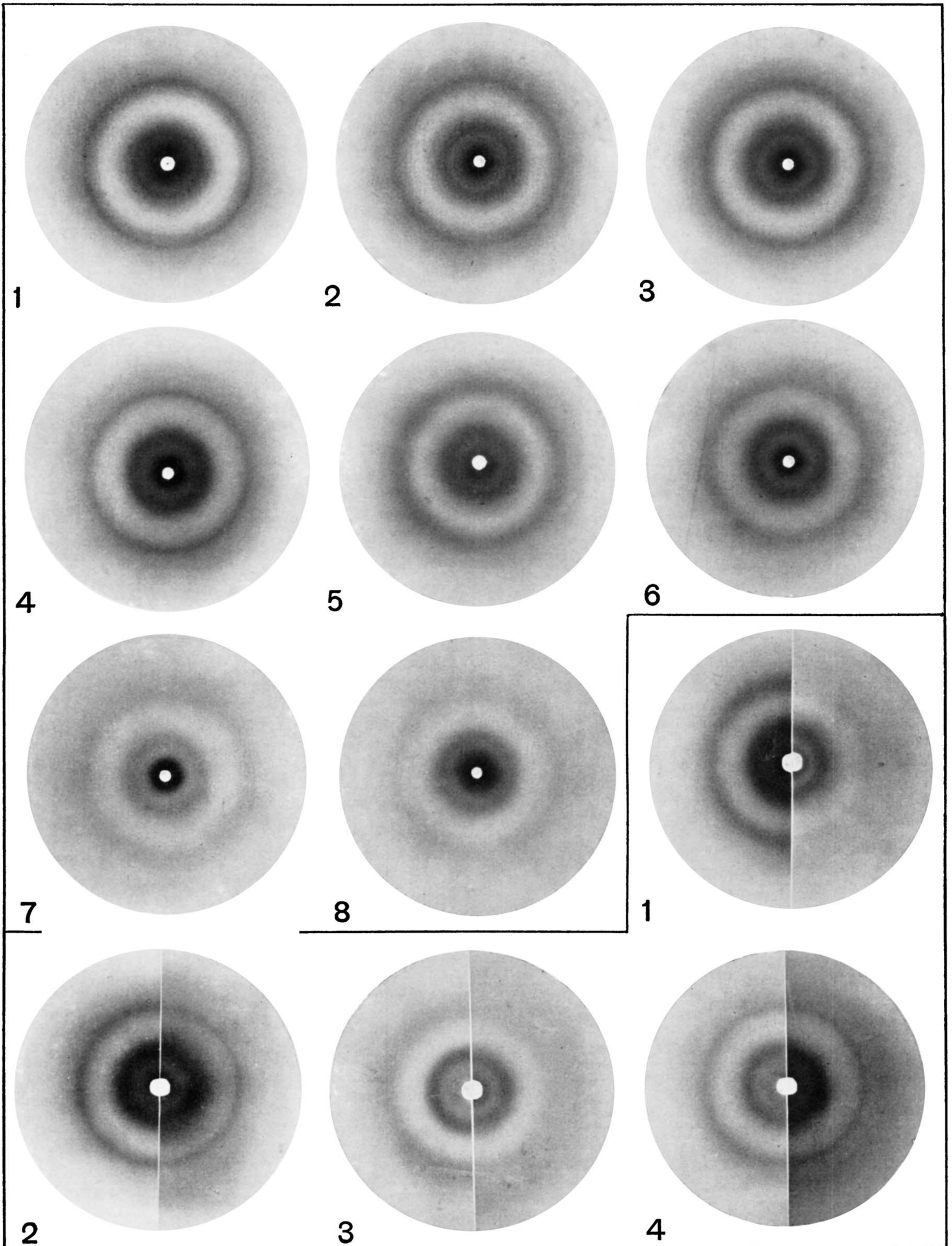


Fig. 4.

[To face p. 848.

Fig. 4.

5 6 7

8 9 10

a

b

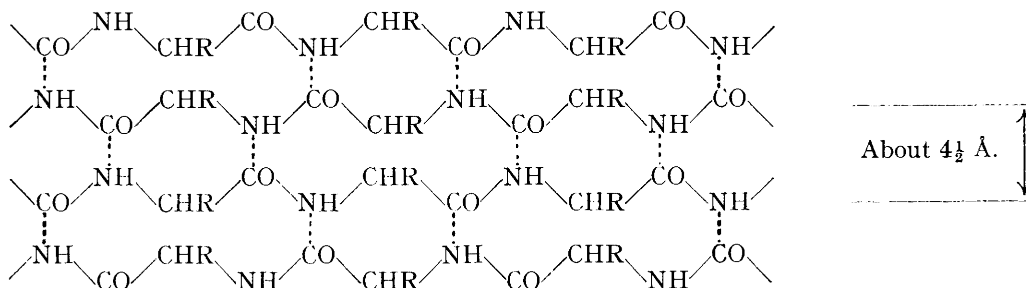
Fig. 2.

B_2 A_2

B_1 A_1

Fig. 5.

peptide chains to be always roughly of the same order, yet we might expect the backbone separation to be almost constant. The backbone linkage should to a certain extent be independent of the nature of the side-chains if the main-chains are fully extended, a reasonable scheme of inter-chain attractions being the following (Astbury and Woods, *loc. cit.*; *Nature*, 1931, **127**, 663; Lloyd, *J. Soc. Chem. Ind.*, 1932, **51**, 141 T):



[N.B. The side-chains (R-groups) must be thought of as standing out above and below the plane of the paper.]

Spacings (Å.U.) *in* X-*ray powder photographs of proteins at ordinary humidity.*

(S = Side-chain spacing; B = backbone spacing.)

	S.					B.			
Tobacco-seed globulin	11·0	$8·6_6$	$7·1_3$	—	$4·9_9$	$4·4_3$	$3·9_5$	$3·5_7$	$3·1_7$
Squash-seed globulin	$11·0_6$	$8·9_5$	$7·2_5$	$5·7_3$	$5·1_4$	$4·4_8$	$3·9_8$	$3·6_3$	3·2
Edestin	11·0	—	—	—	—	$4·5_5$	—	3·6	—
Pepsin	11·5	—	$6·5_5$	—	—	$4·5_9$	—	3·6	—
Denatured egg albumin	$10·2_3$	—	—	—	—	$4·7_5$	—	$3·6_7$	—
Denatured serum albumin	9·6	—	—	—	—	$4·4_8$	—	3·6	—
Egg albumin	$10·6_5$	—	—	—	—	$4·7_5$	—	—	—
Serum albumin	$9·7_1$	—	—	—	—	$4·5_9$	—	—	—
Zein	$9·8_2$	—	—	—	—	$4·6_4$	—	—	—
Casein	$10·2_3$	—	—	—	—	$4·5_9$	—	—	—
Trypsin	$9·8_2$	—	—	—	—	?	—	—	—

The Penetration of Water and Alcohol.—Turning now to the double photographs of Fig. 4, it will be seen that in every case where spacing changes occur on adsorption of water or alcohol vapour, it is the postulated side-chain spacing that increases most, just as expected: the backbone spacing always remains unchanged, or nearly so. Considerable increases are observed in the side-chain spacings of the albumins on exposure to water vapour, and also when zein is exposed to alcohol vapour; but the seed globulins appear to be unaffected by alcohol vapour, and only slightly so by water vapour. The effects shown by pepsin are complex. When first received, the specimen showed also a marked increase in the side-chain spacing on wetting; but on re-examination some months later it was found that wetting produced a new photograph [Fig. 4 (10), right-hand side] which yet always reverted to the original (left-hand side) on drying. In this second photograph of wet pepsin the two main rings are replaced each by a pair of sharper rings.

These swelling results, then, like the spacing measurements quoted above, pronounce definitely in favour of the hypothesis under investigation.

Protein Denaturation (cf., *e.g.*, Lloyd, "Chemistry of the Proteins," 1926, Chap. XIII).—Fig. 5 shows for close comparison the *X*-ray powder photographs of the albumins before and after denaturation by heat. The change accompanying denaturation is simply a sharpening of the backbone reflexion and the appearance of at least one other outer ring of spacing about 3·6 Å. (cf. table): *i.e.*, denaturation of the albumins merely moves them higher up in the "crystallinity series" represented by the photographs of Fig. 3. All these photographs seem to be based on a common structural scheme; in fact, they all appear to arise from crystallites built of parallel, fully-extended polypeptide chains, between which the principal cross-linkages are as illustrated in Fig. 1. Denaturation, then, results in a well-marked improvement in, or development of, this particular mode

of regular aggregation, which is faintly envisaged even in the "poorest" protein photographs, such as those of casein and zein. Before denaturation, the peptide chains of the protein molecule seem to be clinging together chiefly by way of their side-chains, as might be expected (Fig. 1*b*); but after denaturation, this interaction is consolidated by marked coalescence of the main-chains by way of the backbone linkage also (Fig. 1*a*), as is shown clearly by the sharpening of the corresponding *X*-ray reflexion and the appearance of at least one new reflexion.*

To appreciate this result in its proper perspective, it is necessary to consider once more the series of photographs shown in Fig. 3, and to ask ourselves what property, or properties, the proteins there represented have in common. They include obviously very different proteins covering a range of molecular weights, as determined by the ultra-centrifuge, from 34,500 to about 200,000 (see, *inter alia*, Krejci and Svedberg, *J. Amer. Chem. Soc.*, 1934, **56**, 1706), yet they all appear, in varying degrees, to be in a state of similar molecular aggregation—in fact, that state which actually "crystallises out," so to speak, when the albumins are deliberately denatured by heat. The answer to the question must be in three parts as follows: (i) all the proteins photographed are peptides or combinations of peptides; (ii) increasingly marked crystallinity of the type shown in Fig. 3 is an expression of increasingly marked degeneration or denaturation; and (iii) the completely denatured state is that in which the peptide chains have been freed from any specific configuration and aggregated into regular bundles, or crystallites, held together by two principal linkages, the backbone and side-chain linkages illustrated above.

There does not appear to be any difficulty, in view of the source and age of the proteins examined, and also of the fact that they had all been dried, in accepting the argument that they must have been in various stages of degeneration or denaturation: the results presented here can hardly be interpreted in any other way. Only one protein, pepsin, has as yet been analysed by *X*-rays while in the truly active crystalline state (Bernal and Crowfoot, *Nature*, 1934, **133**, 794),† and the photographs obtained are totally different from the pepsin photographs shown in Figs. 3 and 4 (Astbury and Lomax, *ibid.*, p. 795). Indeed, the former are apparently photographs of a regular array of more or less *globular* molecules, in agreement with the molecular shape deduced from the results of experiments with Svedberg's ultracentrifuge (Philpot and Eriksson-Quensel, *ibid.*, 1933, **132**, 932).

From this point of view, the fibrous proteins, such as fibroin, gelatin, keratin, and myosin (Boehm and Weber, *Kolloid-Z.*, 1932, **61**, 269; Astbury and Dickinson, *Nature*, 1935, **135**, 95), may all be looked upon as either actually in a state of denaturation, or configurationally disposed towards such a state. Fibroin and keratin are very insoluble proteins, and so is myosin when once it has been thoroughly dried, and even gelatin becomes progressively more insoluble on standing. At all events, we appear to have a significant analogy between these characteristically stable proteins and non-fibrous proteins which have been brought into a stable and resistant state by one or other of the methods of denaturation. One of the simplest of these methods is the act of dehydration—*e.g.*, the globulins are susceptible in this way, while most so-called protein "crystals" in the dry state are probably only pseudomorphs after partial or complete denaturation—and here, at least, the elastic properties of keratin (Astbury and Woods, *loc. cit.*) suggest an interpretation. The intramolecular transformation from α- to β-keratin, or the reverse, is inhibited in the absence of water, for the reason that, unless the active side-chains are solvated, they seize upon one another so strongly that the possibility of intramolecular movement is practically eliminated. Similar ideas also account satisfactorily for the well-known hysteresis between the adsorption and desorption of water by proteins, while for stability to be maintained in regular aggregations (crystals) of "globular" protein molecules we can imagine that it is essential for each molecule to be "protected" from its

* The side-chain reflexion being called (001) and the backbone reflexion (200), as in β-keratin (Astbury and Street, *loc. cit.*), this reflexion of spacing about 3·6 Å. would appear to correspond to (210), perhaps slightly confused with (020).

† [Note added in proof.] Crystalline insulin has now been analysed, and similar conclusions drawn (Crowfoot, *Nature*, 1935, **135**, 591).

neighbours by an intermolecular wrapping of water or some still more specific reagent. Once the system of protective molecules becomes inadequate, *e.g.*, owing to evaporation, neighbouring protein molecules may powerfully disturb or actually destroy one another, with the result that their constituent peptide chains are liberated to recrystallise by simple parallel alinement, as described above. If this concept is sound, it means that there are three stages in the life of a non-fibrous protein : (1) the " specific " stage, in which the protein is truly itself (cf. Bonot, *J. Chim. physique*, 1934, **31**, 258) ; (2) the " denatured " stage, in which its delicately balanced configuration has disintegrated sufficiently to release (or even build up) peptide chains; and (3) the " coagulated " stage, when the peptide chains have aggregated into regular parallel bundles, or crystallites, analogous to the normal form of the fibrous proteins, this last stage being naturally facilitated by thermal vibrations (cf. Lloyd, *op. cit.*, p. 228, for the physicochemical distinction between denaturation and coagulation).

Denaturation by simple self-destruction is not, of course, the only means we can conceive by which the specific configuration of a protein may be overturned; in fact, it is more difficult to think of ways of keeping such complex systems stable : hence the variety of methods by which denaturation may be brought about—by heat, mechanical disturbance, radiation, strong acids or alkalis, etc. But, according to the X-ray interpretation presented here, all methods have this in common, that they lead to the break-up of a particular arrangement of peptide chains characteristic of the protein, and prepare the way for reaggregation in stable parallel bundles. If the disintegration of the original configuration involves the dissolution of covalent linkages, then we may expect to find in the denatured product certain new radicals not really characteristic of the protein : in particular, if two peptide chains were originally bound together by sharing cystine residues, hydrolytic breakdown of –S–S– linkages would lead to the appearance of –SH groups (cf. Harris, *Proc. Roy. Soc.*, 1923, *B*, **94**, 426 ; Speakman, *Nature*, 1933, **132**, 930).

One final point is noteworthy. In Fig. 4, (1) and (2), and (3) and (4), it will be seen that the side-chain spacing of the albumins, after denaturation, is somewhat less susceptible to water adsorption : and again, in the table, it will be seen that the actual value of the side-chain spacing appears to be rather smaller after denaturation. In other words, not only has denaturation greatly favoured the backbone linkage, but it has also " tightened " the side-chain linkage and decreased its capacity for separation on the entry of water molecules. This also might reasonably be expected from the present standpoint, for the special configuration of an undenatured protein would not necessarily involve the closest possible approach or mutual saturation of the constituent peptide chains : rather would a certain looseness and freedom of active radicals be more probable. Denaturation, on the other hand, would put an end to all that by shaking the chains apart, bringing them into more intimate contact, and effectively masking many of the properties they might otherwise manifest.

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