97. X-Ray Diffraction Studies in Iodinated Amino-acids and Proteins

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The biological activity of some of the iodinated amino-acids and proteins gives rise among others to the additional problem 'does the process of iodinating the protein also change molecular configuration so as to produce physiological activity?' [Salter, 1940]. X-ray diffraction studies of the various stages of iodination in aromatic amino-acids and in proteins seemed therefore promising.

Tyrosine and thyroxine and some of the intermediate products were investigated. Natural iodinated globulins, thyroglobulins, were made available to us through the kindness of Dr J. F. McClendon. Besides, we were able to study an artificially iodinated protein, iodocasein, which according to findings of Ludwig & Mützenbecher [1939], confirmed by Harington & Pitt Rivers [1939], is physiologically active and contains thyroxine. Finally, a plastein derived from the above iodocasein has been prepared by one of us (R. H. H.).

Technique and material

The X-ray diffraction apparatus used in our measurements has been described before [Spiegel-Adolf & Henny, 1939]. The only modification of the method was made necessary by the varying content of iodine with its high opacity to X-rays. Although the iodine content practically did not interfere with the X-ray diffraction of natural and artificial iodoproteins, it made analogous studies impossible in iodinated aromatic amino-acids. Therefore, following Clark [1940], admixtures of gums (tragacanth and acacia) were used, corresponding to a ratio of 8 : 1 in terms of iodine. The X-ray diffraction patterns of tragacanth and acacia are different, so that by using either one in different exposures, besides taking control diffraction patterns with pure gums, it is easy to eliminate diffraction lines pertaining to the gums. A uniform exposure time of 2 hr. was used.

No comparable data seem to be available for our iodinated material. Therefore KI, the powder data of which have been reported by Hanawalt, Rinn & Frevel [1938], was studied in an appropriate mixture with charcoal. In Table 1 our results are presented in comparable form with those of Hanawalt *et al.* They seem to agree sufficiently.

Table 1. *KI 1 part, charcoal 6 parts. Comparison of molecular spacings*

Line, in order of intensity	Our value Å.	Published value Å.	Line, in order of intensity	Our value Å.	Published value Å.
1	3.525	3.53	4	2.147	2.13
2	2.520	2.50	5	2.045	2.03
3	4.09	4.08			

3:5-Diiodotyrosine was prepared by direct iodination of *l*-tyrosine (Eastman) in strong ammonia solution at 0° with the theoretical amount of iodine in KI solution; the material was recrystallized from 33 % acetic acid and finally from water. The iodine content is 58.7 %.

3:5-Diiodothyronine was prepared by the method of Harington & Barger [1927] as modified by Harington & McCartney [1927]. After recrystallization from alcohol the m.p. was 241°, and the iodine content 48.3 %.

Two different commercial brands of thyroxine were used, a preparation of natural thyroxine (Squibb) and a synthetic preparation from Roche. Three samples of human thyroglobulins were available which had been prepared from the original glands by Cavett, Rice & McClendon [1935] by the method of Cavett & Seljeskog [1933]. The origins of our thyroglobulin preparations, their iodine, thyroxine and phosphorus content as originally evaluated are summarized in Table 2.

Table 2. *Data on origin and chemical analyses of thyroglobulins [Cavett, Rice & McClendon]*

Thyroglobulin from	Colloid goitre	Exophthalmic goitre	Normal gland
Thyroxine %	0.0046	0.153	0.382
Total iodine %	0.031	0.40	0.57
Thyroxine iodine			
Total iodine	0.11	0.25	0.45
Phosphorus %	0.032	0.035	0.025

These preparations still contained amounts of ammonium sulphate which interfered with the X-ray diffraction patterns. Part of the preparations were freed from the salt contaminations as described by Cavett & Seljeskog [1933] using heat precipitation, and subsequent washing of the precipitates. To avoid denaturation the remaining preparations were dissolved in water, dialysed against flowing distilled water and finally electro-dialysed until free from electrolytes. The preparations were then dried in a desiccator over P_2O_5 and ground in an agate mortar. Iodinated casein was prepared by direct iodination of casein [Pfanstiehl, Casein Pure, lot 552]. The casein was dissolved in strong ammonia (sp. gr. about 0.93). The solution was cooled to 0° and with good stirring the amount of strong aqueous I_2 -KI was added that was calculated to be required for complete iodination of all tyrosine groups. The ammonia was evaporated off in a vacuum, and the iodinated product was reprecipitated several times at its isoelectric point. (Found: I, 5.2; N, 12.78%; I/N ratio 0.407.) Assuming N 15.7% and tyrosine 4.5% for casein, the theoretical I/N ratio is 0.401. Plastein from iodinated casein was prepared in a manner similar to that of Wasteney & Borsook [1924-5]. Iodinated casein was dissolved in dilute HCl (pH 1.96) and digested with pepsin. The acidity was adjusted to pH 4.0, and insoluble material filtered off. The solution was concentrated at low temperature, and a large amount of pepsin in concentrated solution was added. The precipitate that developed during 2 hr. at 60° was washed three times in the centrifuge with water and then dried. (Found: I, 6.74; N, 11.81%; I/N ratio = 0.571.)

Results

The findings of X-ray diffraction studies in iodinated aromatic amino-acids and derivatives are summarized in Table 3.

These results show that iodination of tyrosine and diiodothyronine notably alters the X-ray diffraction patterns of these compounds. Two diffraction rings (corresponding to spacings of 4.35 and 5.03 Å. respectively) are strong in the tyrosine and weak in the diiodotyrosine diffraction patterns. Conversely at least two new diffraction rings (corresponding to spacings of 3.92 and 13 Å.) become manifest in diiodotyrosine and persist in diiodothyronine and thyroxine. One of these rings (corresponding to a spacing of 3.92 Å.) may have direct relation to iodine since it not only exists in the diffraction patterns of the above mentioned organic compounds but also in mixtures of tragacanth and KI, but not in mixtures of KI and charcoal. The diffraction pattern of diiodothyronine contains many more rings than the one of thyroxine. A moderately strong ring corresponding to a spacing of 5.57 Å., and two faint rings corresponding to spacings 2.13 and 2.36 Å., which occur in the former compound are missing from the thyroxine

Table 3. *X-ray diffraction patterns of iodinated aromatic amino-acids and controls*

(Interplanar spacings in Ångström units, lines in order of intensity.)

	Tyrosine*	Diiodo-tyrosine	Diiodo-thyronine	Thyroxine		Tragacanth	KI
1	4.35	3.92	5.57	3.97	1	9.78	3.53
2	5.03	12.97	13.72	12.28	2	5.22	2.50
3	9.78	3.03	3.86	3.57	3	4.22	3.92
4	5.69	5.03	4.22	3.30	4	—	2.15
5	2.13	4.35	4.75	4.36	5	—	2.05
6	2.28	7.86	9.78	5.02	6	—	2.75
7	3.12	2.78	3.06	4.66			
8	2.67	3.41	2.63	2.67			
9	3.33	2.55	3.62	—			
10	—	—	3.33	—			
11	—	—	2.36	—			
12	—	—	2.13	—			

* Tyrosine contains the same amount of tragacanth as diiodotyrosine.

diffraction pattern. Conversely the latter pattern only shows a strong ring corresponding to a spacing of 3.57 Å. These findings seem to indicate that iodination of aromatic amino-acids is probably accompanied by some kind of molecular rearrangement.

Neither the naturally iodinated thyroglobulins nor the artificially iodinated casein show any differences in the X-ray diffraction patterns which would distinguish them from the corresponding iodine-free proteins. In both instances the two diffuse rings correspond to spacings of 4.5 and 10.19 Å. Thyroglobulin was especially compared with pseudoglobulin which is precipitated by similar ammonium sulphate concentrations. On account of its water-solubility Heidelberger & Pedersen [1935] call thyroglobulin a pseudoglobulin. The undenatured thyroglobulins show diffraction patterns free from any back-bone sharpening. (According to Spiegel-Adolf & Henny [1941; 1942] these findings do not imply that the thyroglobulin is necessarily undenatured; they only suggest that a denaturation is possibly different from changes brought about by heating.) The well-known heat-precipitability of thyroglobulin is accompanied as in other proteins by a sharpening of the back-bone reflexion. This seems interesting in view of the large molecular weight of thyroglobulin [Heidelberger & Pedersen, 1935], which apparently does not interfere with the process of heat-denaturation.

Neither of the two groups of iodinated proteins shows any diffraction rings found in the X-ray diffraction patterns of diiodothyronine or thyroxine. It seems probable that the small amount of thyroxine present in thyroglobulins (even those richest in thyroxine do not contain over 0.38 %) may account for these negative findings. In order to test this assumption X-ray diffraction pictures were taken of artificial mixtures of thyroglobulins and thyroxine containing 4, 2 and 1 % of the latter. While samples containing 4 % thyroxine showed clearly some of the thyroxine diffraction lines, especially the ones corresponding to spacings of 3.57 and 3.97 Å., only traces of these lines could be recognized in the 2 % sample. The 1 % sample did not show any indications of thyroxine admixtures. We do not suggest that our mixtures are identical with physiological compounds of higher thyroxine content. Nevertheless it seems probable that the X-ray diffraction method as handled by us is not sensitive enough to trace minute amounts of thyroxine present in normal and pathological thyroglobulins.

The negative findings in the case of iodocasein were perhaps even more surprising in view of the high iodine content of our preparation. Assuming all the iodine to be in the form of diiodotyrosine, an iodine content of 5.2 % corresponds to a diiodotyrosine content of 8.87 %. Artificial mixtures of this amount of diiodotyrosine and casein show very clearly the characteristic diffraction pattern of the former substance superimposed on the protein diffraction pattern. It seems therefore that the molecular arrangement of the iodocasein is such as to prevent the spacings of the free diiodotyrosine from becoming

manifest. A similar changed state or relationship of the atoms may well bear on the question raised by Harington & Pitt Rivers as to how it is possible for thyroxine to be produced by iodination of a protein and subsequent hydrolysis. Such a synthesis is impossible in vitro by action of iodine upon thyronine.

Our method was more successful in tracing differences existing between plastein derived from iodocasein and its mother-substance. According to our results plastein shows that the diameter of the inner diffraction ring, corresponding to the side-chain spacing, is 2 mm. smaller than the inner ring of the iodocasein diffraction pattern. Besides, the outer diffraction ring, corresponding to the back-bone spacing, is sharp in plastein and diffuse in iodocasein. In order to get an objective evaluation of these differences, relative light transmission graphs and exact centre determinations of these films were made with the help of our photoelectric microdensitometer [Spiegel-Adolf & Peckham, 1940]. The differences observed in the X-ray diffraction patterns of plastein can be probably explained by differences in hydration [Katz, 1934]. Our plastein shows appreciable physiological activity and chemically a much higher thyroxine content than iodocasein. Salter [1935] who studied a plastein resynthesized from thyroid peptones could at least trace differences of physicochemical nature between the artificial and the original products. Our findings seem to add additional material to the arguments of authors, who like Flosdorf [1941], deny the identity of plasteins with their mother substance.

SUMMARY

1. X-ray diffraction studies were made of tyrosine, 3:5-diiodotyrosine, 3:5-diiodo-thyronine, thyroxine, thyroglobulins of human origin, iodocasein, and plastein from iodocasein.

2. Differences were revealed in the X-ray diffraction patterns of the various iodinated amino-acids, indicating some kind of structural rearrangement in the course of iodination.

3. The X-ray diffraction patterns of thyroglobulins of varying thyroxine content and of iodocasein are identical with the X-ray diffraction patterns of the corresponding iodine-free proteins. In artificial mixtures a content of 2% thyroxine seems to be the lower limit for detection by X-ray diffraction methods. Diiodotyrosine admixtures corresponding to the iodine content of iodinated casein are plainly visible in the X-ray diffraction pattern of the casein-diiodotyrosine mixtures.

4. Plastein made from iodocasein shows X-ray diffraction patterns different from those of the latter substance.

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