LIV. THE ULTRAVIOLET ABSORPTION OF SHEEP THYROGLOBULIN

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ANALYSIS of the ultraviolet absorption curve of thyroglobulin in terms of the absorption of its iodine-containing components thyroxine and diiodotyrosine leads to interesting results. The absorption spectra of these amino-acids have previously been measured by Hicks [1925], Abderhalden [1927] and Heidt [1936]. In Fig. 1 our own results are given. The measurements were made on alkaline solutions (0.02 *N* NaOH) and acid solutions (diiodotyrosine in 0.1 *N* HCl; thyroxine in 0.014 *N* HCl). At the *pH* of the alkaline solutions ($pH \geq pK + 2$) the phenolic hydroxyl, the ionization of which governs the change of absorption spectrum from the acid form to the alkaline form [Stenström & Reinhardt, 1925, 2], is almost completely ionized whereas in the acid solutions ($pH \leq pK - 2$) it is almost completely unionized. For diiodotyrosine

$$pK = 6.2.$$

In these absorption curves no "fine structure" (vibrational structure) is present. We cannot, therefore, expect to be able to detect the presence of thyroxine and diiodotyrosine in thyroglobulin by characteristic peaks in the thyroglobulin curve. This conclusion is confirmed by the smoothness of the absorption curves of thyroglobulin at *pH* 8.0 and *pH* 1.3 (Fig. 2). The curves show a single maximum at 280 *mμ*—the well-known protein band due to tyrosine and tryptophan. No maximum can be distinguished in the region of the alkaline absorption bands of thyroxine and diiodotyrosine from 350 to 300 *mμ*.

Two methods of quantitative analysis are possible. First, one can compare the thyroglobulin absorption curve at *pH* 8.0 with that of serum globulin at *pH* 8.0 (curve 2, Fig. 2). This method is open to objections, e.g. the difference between serum globulin and the globulin in thyroglobulin. A second method has proved to be more satisfactory. Here the different compounds are not characterized in the usual way by an absorption curve which represents the (molecular) extinction coefficient as a function of the wave-length, but by a curve which represents the so-called differential (molecular) extinction coefficient as a function of the wave-length.

Fig. 1. Molecular extinction coefficients of: 1, thyroxine in 0.02 *N* NaOH; 1A, thyroxine in 0.014 *N* HCl; 2, diiodotyrosine in 0.02 *N* NaOH; 2A, diiodotyrosine in 0.1 *N* HCl.

The differential extinction coefficient δ at a given wave-length is defined as the difference between the alkaline extinction coefficient K (at $pH \geq pK + 2$) and the acid extinction coefficient K' (at $pH \leq pK - 2$). In a similar way the differential molecular extinction coefficient δ_ϵ is defined as the difference between the alkaline molecular extinction coefficient ϵ and the acid molecular extinction coefficient ϵ' .

The advantage of the use of δ instead of K or K' lies in the fact that δ is zero for all compounds which show no shift in the absorption spectrum in the given pH range.

Fig. 2. 0.7% thyroglobulin solution at pH 8.0 (1) and at pH 1.3 (1A). 1.0% sheep serum globulin (2).

By means of the δ curves it has been possible to analyse the thyroglobulin curves in the region 400–300 $m\mu$. An extension of the analysis to wave-lengths below 300 $m\mu$ failed because of masking by the protein bands. In Fig. 3 are

Fig. 3. 1, Molecular extinction of thyroxine; 2, molecular extinction of diiodotyrosine; A, in alkali; B, in acid; C, molecular differential extinction.

represented the δ_ϵ , ϵ and ϵ' curves of thyroxine (1) and diiodotyrosine (2). The values of the molecular (differential) extinction coefficients are given in Table I.

As may be noticed, the δ_ϵ bands are more sharply defined than the ϵ bands and for this reason they are more apt to characterize the compounds. The following is a description of the way in which the δ curve of the iodine compounds

Table I

Thyroxine							
λ (m μ)	360	350	340	330	320	310	300
$10^{-3} \epsilon$	1.1	2.6	5.0	6.1	6.1	4.9	3.4
$10^{-3} \epsilon'$	—	—	0.1	0.4	2.1	3.9	5.0
$10^{-3} \delta_e$	1.1	2.6	4.9	5.7	4.0	1.0	-1.6
Diiodotyrosine							
$10^{-3} \epsilon$	—	—	0.8	2.4	4.9	5.6	4.5
$10^{-3} \epsilon'$	—	—	—	—	—	0.3	1.5
$10^{-3} \delta_e$	—	—	0.8	2.4	4.9	5.3	3.0

in thyroglobulin can be derived from the absorption curves of thyroglobulin at pH 8 and pH 1.3.

In the region from 400 to 300 m μ the absorption K_t of thyroglobulin is determined by the following factors (Fig. 2, curves I and IA):

- Scattering of light: K_s (present from 400 to 300 m μ).
- Absorption of the iodine components K_i (present from 370 to 300 m μ).
- Absorption of the globulin K_g (present from 320 to 300 m μ).

Each of these quantities will be discussed briefly.

K_s is caused by the presence of larger protein particles in the solution which cannot be centrifuged down even at 15,000 rev./min. The properties of this scattering have been studied in solutions of serum globulin (Fig. 2, curve 2); there is no reason why the scattering of serum globulin should differ much from the scattering of thyroglobulin. The serum globulin used was prepared in a way similar to that used for the preparation of thyroglobulin.

The scattering was found to be proportional to $1/\lambda^4$. Thus the scattering could be described by the Rayleigh-Jeans formula for the scattering of particles which are small compared with the wave-length of the incident light.

K_i occurs below 380 m μ in the thyroglobulin curve. Above this wave-length the thyroglobulin curve is similar to the serum globulin absorption curve (Fig. 2, extinction coefficient proportional to $1/\lambda^4$). Below this wave-length the thyroglobulin curve increases more rapidly than the serum globulin curve owing to the occurrence of K_i in the thyroglobulin curve. K_g is only of importance below 320 m μ [Gróh & Hanák, 1930]. The value of K_g is the same at pH 8 and at pH 1.3. K_g increases first at pH values above 10 [Stenström & Reinhardt, 1925, 1]. Now we can write that at pH 8: $K_t = K_s + K_i + K_g$ and at pH 1.3: $K_t' = K_s' + K_i' + K_g'$. Because $K_g = K_g'$ we can deduce that

$$K_i - K_i' = (K_t - K_t') - (K_s - K_s').$$

The left-hand member of this equation represents the differential absorption δ_i of the iodine compounds. The values of K_t and K_t' present in the right-hand member are determined experimentally.

K_s and K_s' are only known at wave-lengths above 370 m μ (here K_i and K_g are both zero, the absorption is only due to K_s). At those wave-lengths $K_s = K_t$ and $K_s' = K_t'$. By application of the Rayleigh-Jeans formula, we can find the values of $K_s - K_s'$ below 370 m μ by extrapolation. If these values are calculated by extrapolation of the value at 380 m μ , which value is denoted by $(K_t - K_t')_{380}$, the formula for $K_i - K_i'$ or δ_i becomes

$$\delta_i = (K_t - K_t') - (K_t - K_t')_{380} \times (\lambda/380)^4.$$

In the deduction of this formula we have assumed that pH 8 and pH 1.3 lie outside the interval from $pK - 2$ to $pK + 2$. That the assumption is sound was verified by the measurement of K_i at different pH values (pH 8.0, 6.8, 6.0) and calculation of the values of $K_i - K_i'$. From these data a value between 5 and 6

was deduced for the pK value of the iodine compounds in thyroglobulin by means of a well-known formula from the theory of dissociation. Therefore pH 8 and 1.3 lie on either side of $pK - 2$ and $pK + 2$.

By means of equation (1) the δ curve of the iodine compounds present in thyroglobulin was deduced from the absorption curves of solutions of sheep thyroglobulin (for the preparation of solutions see below under (b)).

The shape of these δ curves agreed fairly well with the curves which could be calculated if half of the iodine in the thyroglobulin solution was attributed to thyroxine, the other half to diiodotyrosine. Moreover, the values of the total iodine per ml. of solution which were obtained from the δ curves did not differ much from the values which were obtained by direct chemical analysis:

	1	2	3	4	5	6
Iodine per ml. of solution : δ curves	36	38	36	45	47	49
Iodine per ml. of solution : chemical analysis	38	42	39	47	49	52

1, 2, *Elityran* (a liquid thyroglobulin preparation of I.G. Farbenindustrie) after extraction with ether; 3, own preparation of thyroglobulin; 4, 5, 6, thyroglobulin I.G. Farbenindustrie.

The δ curves of the iodine compounds in thyroglobulin which were measured for two solutions (of different samples of thyroglobulin) are reproduced in Fig. 4, curves *A* and *B*. The error in the δ values is 3% (error in extinction 1.5%). Accidentally the δ curves coincide at 320 $m\mu$. Now these curves must be compared with the δ curves which can be calculated for a thyroxine-diiodotyrosine mixture containing two diiodotyrosine molecules to one thyroxine molecule (iodine equally distributed over thyroxine and diiodotyrosine). This calculated curve is represented in Fig. 4, curve 2. Besides the condition mentioned (thyroxine-iodine = diiodotyrosine-iodine), the total quantities of thyroxine and diiodotyrosine are chosen in such a way that the calculated curve coincides at 320 $m\mu$ with the experimental curves.

Fig. 4. Experimental δ curves (*A* and *B*). Mean values of *A* and *B* (curve 1). Theoretical curve based on ratio of thyroxine-iodine to diiodotyrosine-iodine of 1 : 1 (curve 2); 4 : 6 (curve 3); 6 : 4 (curve 4).

It may be seen that the agreement between calculated and experimental curves is satisfactory. If we compare the total iodine per ml. of solution, which can be deduced from the curve, with the chemical values (as has been done above for six other solutions) we find 55 $\mu g.$ per ml. of solution from the δ curve whereas the chemical values are 55 $\mu g.$ and 51 $\mu g.$

In curves 3 and 4 (Fig. 4) the lack of agreement between calculated and experimental δ curves is demonstrated if we base the calculation on 40% thyroxine-iodine (curve 3) and on 60% thyroxine-iodine (curve 4). Under these conditions a considerable difference in shape between the calculated and experimental curves (curve 1) is shown. Therefore the value of the diiodotyrosine-

iodine/thyroxine-iodine ratio may be determined with fair accuracy by means of an analysis of the δ curve. This ratio cannot differ much from unity in the seven samples of sheep thyroglobulin which have been studied here.

It seems interesting to check whether the same value for this ratio is valid for other kinds of thyroglobulin (ox, hog). As a first experiment the absorption of a solution of ox thyroglobulin was examined (0.7% solution of ox thyroglobulin, I.G. Farbenindustrie).

The experimental δ curve agreed with the δ curve of a diiodotyrosine-thyroxine mixture which contains 50% thyroxine-iodine. The δ curve corresponded to 21 μg . iodine per ml.; the chemical analysis yielded 24 μg . iodine per ml.

These data do not agree with experiments of Leland & Foster [1932], or of Abelin [1932] who found a mean value of about 0.4 for the ratio diiodotyrosine-iodine to thyroxine-iodine in thyroglobulin. Our results seem to agree better with determinations of thyroxine-iodine which have been carried out by Harington & Randall [1929], and Rotter & Mecz [1932]. Rotter & Mecz found a mean value of 1 for the diiodotyrosine-iodine/thyroxine-iodine ratio (19 different thyroglobulin preparations). However, their values vary from 1.4 to 0.4. Our values do not show such variations. This may be explained by the relatively small number of samples (8) which have been studied by us, but will be verified in further experiments.

Here the disadvantage of chemical methods used for the determination of the thyroxine content of thyroglobulin, i.e. changes of the chemical structure, is avoided. On the other hand, the absorption method can only be used for thyroid preparations which yield a clear solution at pH 8 and pH 1.3. The extinction coefficient at 400 $m\mu$ is below 0.7 if iodine concentration is 20 μg . per ml. of solution.

We may conclude that this spectrographic analysis offers strong support for the conception that $-\text{C}_6\text{H}_2\text{I}_2\cdot\text{O}\cdot\text{C}_6\text{H}_2\text{I}_2\text{OH}$ groups (chromophoric group of thyroxine) and $-\text{C}_6\text{H}_2\text{I}_2\text{OH}$ groups (chromophoric group of diiodotyrosine) exist in the thyroglobulin molecule. The absorption is mainly due to these chromophoric groups and not to the whole molecule; hence we must not deduce the occurrence of thyroxine and diiodotyrosine as such in the thyroglobulin molecule; changes in the side chains of these iodine compounds remain possible.

Some attention may be paid to the small difference in shape between the experimental and calculated curves. The accuracy of most of our measurements of the δ curve have a mean error of 6%. However, the experimental curves A, B of Fig. 4 were determined with the utmost care (see below under *d*). Therefore we must accept the reality of the small differences between these curves and the theoretical curve (curve 2, Fig. 4).

SUMMARY

1. An analysis of the thyroglobulin absorption curve in the region from 400 to 300 $m\mu$ is given. In this analysis differential extinction coefficients have been used. This quantity is defined as the difference between the extinction of the solution if practically all the absorbing molecules have the alkaline form (pH above $pK+2$) and the extinction of the solution if practically all the absorbing molecules occur in the acid molecule-form (acid absorption, pH below $pK-2$).

2. The differential absorption curve of the iodine compounds, as deduced from the absorption curves of (sheep) thyroglobulin at pH 8 and 1.3, gives strong evidence for the presence of the $-\text{C}_6\text{H}_2\text{I}_2\text{OH}$ group (chromophoric group of diiodotyrosine) and the $-\text{C}_6\text{H}_2\text{I}_2\cdot\text{C}_6\text{H}_2\text{I}_2\text{OH}$ group (chromophoric group of

thyroxine) in the thyroglobulin molecule; two of the former groups occur to one of the latter groups.

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APPENDIX

(a) Determination of the acid absorption of thyroxine. A first attempt to determine the acid thyroxine absorption directly failed (absorption tube of 120 cm. filled with saturated thyroxine solution in 0.1 *N* HCl). Therefore we made use of the adsorption of thyroxine on to a substance which remained soluble at pH 1.3.

Dr W. M. Bendien in this laboratory has shown that such a protective colloid is present in serum (horse, human, sheep). He found that thyroxine which is perfectly ultrafiltrable in its solution in 0.02 *N* NaOH becomes non-ultrafiltrable if dissolved in human serum: evidently thyroxine is adsorbed by one of the constituents of the serum (? globulin). With this fact in mind it was easy to get (adsorbed) thyroxine into acid solution.

2 ml. horse serum are mixed with 8 ml. of a 0.066% thyroxine solution (0.02 *N* NaOH). Then 0.3 ml. of *N* HCl is added. In this way the solution is brought to pH 1.8 without any precipitation of thyroxine. By subtracting the absorption of a mixture containing 2 ml. horse serum, 8 ml. of 0.02 *N* NaOH and 0.3 ml. of *N* HCl from the absorption of the former solution, the absorption of the thyroxine in acid solution was obtained. Adsorbed thyroxine and free thyroxine show the same absorption, as could be verified in alkaline solution; no appreciable difference could be found between the absorption of a thyroxine solution in 0.02 *N* NaOH and the absorption of a thyroxine solution in alkaline serum.

(b) Thyroglobulin is not easily dissolved if directly treated with acid. In order to obtain an acid solution, the thyroglobulin must be dissolved first in an alkaline solvent. The borate buffer solution of pH 8 may not be used as a solvent: if this solution is made acid by adding *N* HCl solution, a small part of the thyroglobulin is precipitated. This is not the case if the thyroglobulin is dissolved in 0.02 *N* NaOH. Thus thyroglobulin solutions at pH 8 and 1.3 which may be used for the absorption analysis are obtained by first preparing a solution of thyroglobulin in 0.02 *N* NaOH (about 8 mg. thyroglobulin per ml.): One part of this solution is acidified by adding quickly *N* HCl till the pH reaches 1.3, whereas to the other part concentrated buffer-solution is added to bring the pH to 8.

(c) The photographic method which was used for the determination of the absorption curves (except those reproduced in curves *A* and *B*, Fig. 4) has been described previously [Ginsel, 1936]. Because this method is based on the use of density marks, the absorption coefficients can be determined at any given wavelength. Its simplicity is due to the fact that only those transparencies of the photographic plate are used which correspond to the straight part of the transparency curve (transparency is intensity of light transmitted by exposed area divided by intensity of light transmitted by unexposed area; transparency curve is transparency plotted against log of relative intensity).

It is stated [Ginsel, 1936] that transparencies between 0.35 and 0.65 correspond to the straight part of the transparency curve. After a longer experience with

Ilford Auto filter plates (400 H and D) we have found however that small deviations from straightness may occur sometimes in this transparency region. The only part of the transparency curve which shows perfect straightness in all the cases is located between transparencies 0.45 and 0.65.

If we are limited to the use of this transparency region, it is only possible to obtain a complete absorption curve if the thicknesses of the liquid layers increase by a factor not greater than 1.7. Unfortunately our tubes increase by a factor 2. Therefore we could not avoid the use of transparencies lying outside the range 0.45–0.65. In this way small errors (below 3%) may have been introduced in most of our absorption curves.

In the determination of curves *A* and *B*, Fig. 4, we did not use the simple method developed by us but reverted to the actual use of density curves, in order to reduce sources of errors. The absorption coefficients were deduced from the densities on the photographic plate in a way similar to the well-known procedure used in photographic intensity measurements.

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