GLYCOPEPTIDES IN THE THYROID GLAND OF RATS

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Levine [1949] has shown that a ‘spreading factor’, or mucolytic activity, is present in large amounts in the thyroid gland of rats and guinea-pigs and that the enzymic activity varies according to the physiological state of the gland. These findings made it desirable to identify the substrate, and to decide whether its concentration in the colloid also varied in different states of activity. A solution to the problem became possible with the development by Hotchkiss [1948] of a procedure for visualizing polyelectrolytes. A complicating factor, which was also investigated, is that purified thyroglobulin is rich in carbohydrates, of which glucosamine is the chief component [Brand, Kassell & Heidelberger, 1939; Salter, 1940]. As expected, this protein gave a positive spot test with the periodic acid-leucofuchsin method and its presence in colloid would suggest that at least two groups of reacting substances might be found there. Two such substances have now been identified and ‘separated’. The concentration of reacting substances in each has been measured in numerous follicles in different states of activity and a search made for precursors in the gland cells. Additional observations on the ground substance of the connective tissue and basement membrane are described in another paper [Gersh & Catchpole, 1949].

MATERIAL AND METHODS

The thyroid glands of rats were removed rapidly after sudden death and fixed by freezing in liquid air (first series) or in isopentane at approximately −150° C. (second series). They were then embedded in paraffin and sectioned at 4 µ for the measurement of total glycoprotein, at 2, 4, and 8 µ for cytochemical study, and at 10 µ for solubility studies and the measurement of residual protein. The sections were mounted with light pressure on slightly albuminized slides. Sections studied for the measurement of total glycoprotein were passed through petroleum ether and immersed in absolute alcohol for several hours, and were then stained by the Hotchkiss procedure. The depth of the purplish red colour represents the amount or number of reacting groups present in the colloid, and was measured in the colloid of individual follicles by using monochromatic light with a maximum at about 550 mµ. This was achieved by interposing three Corning glass filters (512, 430 and 350) between the medium pressure mercury arc lamp used as a light source and the substage prism. For most measurements, light transmitted by an area of colloid 9.1 µ in diameter was measured by the use of 8 × ocular and 16 mm. apochromatic objective, with a magnification of ×430 at the aperture of the electron multiplier tube. In a few glands, where the lumen of the

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follicles was small, a 4 mm. apochromatic objective was used with a corresponding decrease in the area of the field measured. The light transmission was recorded by the deflexion of a galvanometer mirror on a scale, and compared with complete transmission to yield the percentage transmission and hence absorption. As the blank reading on unstained colloid was small, this was ignored in calculation of percentage absorption. Serial sections were used for all measurements to minimize errors arising from variations in thickness, and not more than two or three follicle-readings were made from each section. About twenty readings were made for each gland.

Sections studied for the measurement of residual glycoprotein were passed through petroleum ether to remove the paraffin, and the solvent was allowed to evaporate in air with slight warming. The sections were covered with five drops of buffer pH 7.0, and precautions were taken to prevent evaporation. After 1 hr. this was decanted and the sections were washed with the same volume of fresh buffer and immersed in alcohol for 16 hr. or more. The residual colloid of the sections was then stained by the Hotchkiss procedure, measured as described above and corrected for difference in section thickness.

Sections studied to ascertain the solubilities of the glycoproteins of colloid were treated similarly, but no absorption spectrum measurements were made. Sections prepared for cytochemical study were also treated similarly, or were stained after passage through xylol and prolonged immersion in absolute alcohol.

The thyroid glands were obtained from two series of rats. The first series consisted of five untreated normal rats, four treated with pituitary extract, four treated with potassium iodide incorporated in the diet, four treated with dietary sulphaguanidine for a period varying from 3 days to 2 weeks, two that were hypophysectomized and three that were hypophysectomized and treated with sulphaguanidine. These were supplemented, for cytological studies only, by the thyroids of seven additional rats treated with pituitary extract and six treated with dietary sulphaguanidine. More detailed information about the treatment of the rats is given in an earlier paper, in which studies on other constituents of the colloid of the same glands are described [Gersh & Baker, 1943]. The second series consisted of normal rats purchased from the Wistar Institute: four were 42 days old, four were 201 days old, and six were 474–829 days old.

RESULTS

As McManus [1946] stated, the colloid of the thyroid-gland follicles is stained brilliantly by the periodic acid-leucofuchs in method. The intensity of the stain is not diminished by extraction of alcohol-treated sections with a hot mixture of equal parts of methanol and chloroform, followed by incubation in saliva. It does not appear after staining with the leucofuchsin without prior oxidation. It is probable, therefore, that the reacting groups of colloid and gland cells are associated with glycoproteins [Gersh, 1949].

By the use of differential solubilities, it was found that the greater part of the reacting or stainable component of the colloid has an isoelectric point at pH 4.5–5.0, i.e. the greater part of the stainable colloid is extracted from sections by buffers at pH 3.6 and 4.0 and by buffers at pH 5.5–10.5. It is soluble also in 1% NaCl at pH 6.3 and 8.0 and in dilute acetic acid solutions at pH 3.3 and 2.75 (0.2 and 2.0 % respectively). It is insoluble in buffers at pH 4.5 and 5.0 and in one-third, one-half
and fully saturated ammonium sulphate solutions. It is digested by pepsin and trypsin, but cannot be extracted by hyaluronidase made up in buffers at pH 5.0. These properties correspond with those of thyroglobulin and it seems probable that most of the reacting groups in colloid are part of this protein.

The residue of stainable colloid components remaining after extraction of the greater portion by reagents described above, has different properties. Though insoluble in all buffers from pH 3.6 to 10.5 it is readily extracted in buffer at pH 11.2. This fraction gives a positive test with Millon's reagent and its ultra-violet absorption curve is that of a non-specific protein, with an absorption peak at 2800 Å. There is no trace of an absorption peak at 3341 Å, indicating that thyroxine and di-iodotyrosine are absent from this protein. Since it is present in unextracted colloid, the organic iodine peaks must be associated with the first, larger fraction, probably thyroglobulin, described above. The second fraction is also digested by pepsin and trypsin, but unlike the first is readily extracted by hyaluronidase dissolved in buffer at pH 7.0.

The evidence shows, therefore, that the colloid contains at least two glycoproteins: one is responsible for most of the reacting groups and resembles purified thyroglobulin; the second may possibly act as a substrate for mucolytic activity. These have been estimated by measurement of absorption by reacting groups of both proteins and of absorption by reacting groups of the second protein after extraction of the first. The difference between these values represents that of the first protein. The values are given as percentage absorption per 4μ section and are useful for comparison of follicular colloid in different states of activity. The measurements and statistical treatment are summarized in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total glycoprotein</th>
<th>Residual glycoprotein</th>
<th>Difference between total and residual glycoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative absorption</td>
<td>P*</td>
<td>Relative absorption</td>
</tr>
<tr>
<td>Normal</td>
<td>81.29±0.40</td>
<td>—</td>
<td>13.9±0.75</td>
</tr>
<tr>
<td>Hypophysectomy</td>
<td>80.07±0.74</td>
<td>0.2±0.1</td>
<td>15.4±1.56</td>
</tr>
<tr>
<td>Potassium iodide in diet</td>
<td>89.71±0.41</td>
<td>0±0.1</td>
<td>18.5±1.33</td>
</tr>
<tr>
<td>Activated by pituitary extract</td>
<td>81.34±0.98</td>
<td>&gt;0.9</td>
<td>20.4±0.68</td>
</tr>
<tr>
<td>Sulphaguanidine in diet</td>
<td>71.46±1.49</td>
<td>&lt;0.01</td>
<td>3.0±0.12</td>
</tr>
<tr>
<td>Hypophysectomy + s.c. in diet</td>
<td>79.84±0.94</td>
<td>0.2±0.1</td>
<td>18.5±0.82</td>
</tr>
</tbody>
</table>

* The probability that the observed effects are due to chance.

It appears that the total reacting groups of the colloid are significantly increased by administration of potassium iodide to rats and decreased by the antithyroid substance sulphaguanidine. The residual glycoprotein of the thyroid colloid appears to respond actively during changes in the physiological state of the gland, with a significant decrease in reacting groups following dietary administration of sulphaguanidine and an increase following the administration of potassium iodide, pituitary extract and hypophysectomy followed by the antithyroid substance. It is not possible to assess statistically the significance of the changes of the presumed thyroglobulin fraction (difference between total and residual glycoprotein values).
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Cytological observations

De Robertis [1942] succeeded in staining with aniline blue two types of 'granules' in the gland cells of the thyroid—one was very small and pale-staining, the other larger and deeper-staining. The larger variety of granules was particularly associated with secretory processes and they will be referred to hereafter as 'droplets' to distinguish them from the smaller granules. Both of these structures are stained by the periodic acid-leucofuchsin method and are presumably glycoprotein. In addition, the cytoplasm appears to be very lightly stained diffusely, outlining the completely unstained nucleus. The homogeneous appearance of the cytoplasmic stain may be referred to submicroscopic particulates equivalent to microsomes described in other cells.

The droplets (Pl. 1, fig. 4) were sometimes visible in the gland cells of normal thyroids, as well as in those of rats treated with sulphaguanidine. They were more numerous and occurred in a larger proportion of cells in the thyroids of animals stimulated with pituitary extract. They were not observed in the gland cells of hypophysectomized animals nor in those treated with iodide. Like the colloid, they give a positive reaction for tyrosine with Millon's reagent and their behaviour toward extractives resembles that of the major fraction of the colloid.

Technical difficulties prevented study of the solubility of the granules which were found in the supranuclear region of gland cells of all animals (Pl. 1, figs. 1–3). The most striking feature appeared in the second series of normal rats of known age. At 42 days granules were present, and at 201 days they appeared in the same region in a larger number of cells. In all older rat glands they were present in the form of a large accumulation of discrete granules of very small size. They seem to correspond very closely with the Golgi apparatus as described by Okkels [1931]. This structure appears to be more 'solid' in his photographs, perhaps because of the deposition and apposition of metal during the impregnation.

DISCUSSION

The results show that there are at least two protein components in the colloid which may change during different states of activity of the thyroid gland. Their relation to the Bauer-positive argyrophilic material described by Dempsey & Singer [1946] is not clear at present. One of the components described above seems to correspond with thyroglobulin and a similar compound appears to be segregated in the cytoplasm as colloid droplets. It is unfortunately not possible to relate the quantitative studies of this fraction of the colloid reported here with earlier ones made on the colloid of the same glands for total protein and organic iodine [Gersh & Baker, 1943]. It is clear, however, that this fraction of the carbohydrate-containing protein material does contain organic iodine. It constitutes the greater part of the reacting groups of colloid.

The smaller fraction, on the other hand, appears to be a glycoprotein whose outstanding quality is its ready extractability by hyaluronidase, which may constitute the substrate for the spreading factor demonstrated in such large amounts in the thyroid gland. The enzyme and substrate compose one system which controls the viscosity of the colloid and thus the availability for reabsorption or retention of the
active principle of the colloid. The other system includes the total protein concentration as affected by catheptic activity [De Robertis, 1941; 1948]. While the secretion of glycoprotein is not affected by hypophysectomy, it is influenced by administration of pituitary extract and by dietary iodide and sulphaguanidine.

It should be pointed out that the work of Levine [1949] and the results recorded in this paper agree on the physical basis of the control of viscosity of the colloid. A corollary is that the colloid is organized on a submicroscopic level, with a structure that may be made manifest by physical methods such as X-ray diffraction.

It appears that the colloid droplets as visualized in rats by aniline blue or as glycoprotein contain segregated thyroglobulin. Using other criteria in guinea-pigs, similar droplets which appeared to contain organic iodine have been described [Gersh & Caspersson, 1940]. Whether or not these are identical is still uncertain.

The smaller granules of the gland cells which occupy a supranuclear position and stain with the periodic acid-leucofuchsin method have been tentatively identified as parts of the Golgi apparatus. If this is so they resemble similar structures in intestinal cells in that both probably consist of glycoprotein [Gersh, 1949]. The reason for the progressive increase in the size of the structure with age is difficult to understand. It may be related to the state of activity of the gland cells. Okkels has shown that the more ordinary Golgi apparatus hypertrophies during hyperactivity of the gland cells.

The smallest particles that react with the Hotchkiss procedure are presumed to be microsomes. The submicroscopic dimensions are considered to be responsible for the homogeneous appearance in sections of a faintly pink tone which is limited to the cytoplasm.

**SUMMARY AND CONCLUSIONS**

1. Thyroid colloid contains at least two protein complexes containing carbohydrate-reacting groups. One appears to be identical with thyroglobulin, while the other, smaller fraction, is responsive to testicular hyaluronidase and may act as its substrate.

2. The mucolytic activity-glycoprotein system is one of the mechanisms controlling the viscosity of the colloid and appears to be significant in the storage or release of hormone from the colloid.

3. Colloid droplets in the cytoplasm of the gland cells behave like the first fraction and may be the segregated precursors of thyroglobulin.

4. Cytoplasmic granules containing glycoprotein may constitute an essential feature of the Golgi apparatus of the gland cells.

5. Submicroscopic particles containing glycoprotein may be present in the cytoplasm.
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REFERENCES

De Robertis, E. [1942]. Anat. Rec. 84, 125.
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DESCRIPTION OF PLATE

Sections of rat thyroid glands prepared by freezing and drying and stained by the periodic acid-leucofuchsin method to show carbohydrate-containing proteins red (black in the photomicrographs). There are two visible kinds of reacting intracytoplasmic materials: the small granules which are near the limit of resolution, and the larger droplets. The first are in relation to the nucleus and may be portions of the Golgi apparatus; the second are unconnected with the nucleus and are secretion antecedents. Both types of material are stainable with aniline blue. The reaction of the colloid is strongly positive; that of the nucleus is always negative. Basement membranes are also visible.

Figs. 1 and 3. Normal rat, 474 days old, with a marked basement membrane, and rich zone of cytoplasmic granules. (× 2140.)

Fig. 2. Young adult rat treated with pituitary extract, with a small number of supranuclear granules. These are found also in normal, unstimulated cells. (× 2140.)

Fig. 4. Young adult rat with cytoplasm containing droplets of reacting material, which vary in size and number from cell to cell. (× 2140.)