The Identification of Thyrotrophin-secreting Cells in the Pituitary Gland of the Minnow (Phoxinus phoxinus)

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With one plate (fig. 2)

SUMMARY

Evidence is given for the existence of two main types of cyanophil cell in the median zone of the glandular lobe (adenohypophysis) of the minnow, distinguishable by their distribution and by their cytological characteristics. Both types are positive to the periodic acid Schiff (PAS) technique, but one (type 2 of this account) also gives a positive response to the aldehyde-fuchsin (AF) technique of Gomori, as used by Halmi and by Purves and Griesbach in studies of the mammalian pituitary. In fish which have been immersed in thiouracil solution the type 2 cells show degranulation and vacuolation, and their characteristic positive AF response is very greatly weakened or lost. For these reasons the type 2 cells are believed to be responsible for the secretion of thyrotrophin, and appear to be very closely comparable with the thyrotroph cells of the pituitary of the rat.

INTRODUCTION

In drawing attention in previous papers to the effect of thiourea in delaying the sexual maturity of the minnow and to the existence of seasonal variation in the heights of the epithelial cells of its thyroid gland (Barrington and Matty, 1952, 1954), we have emphasized the difficulty of interpreting these facts in the absence of adequate information regarding the histophysiology of the pituitary gland of this animal. It has therefore been our purpose in the present work to attempt a demonstration of the source of thyrotrophic hormone (TSH) by employing thiouracil to promote an increased output of the hormone and examining the effect of this upon the pituitary gland, using as histological techniques certain procedures which have recently been applied to this problem in the rat (Purves and Griesbach, 1951). During the course of this study Atz (1953) has published the results of a similar investigation into the pituitary gland of another Teleost, Astyanax. As will be seen, our own results, obtained independently of hers, appear to be in broad agreement with them, but the situation in Phoxinus appears to be even more clear-cut, not only because of the spatial localization of the presumed thyrotroph cells in this fish, but because we have been able to demonstrate a differential response of these to the Gomori aldehyde-fuchsin stain similar to that obtained by Purves and Griesbach in the pituitary of the rat.

METHODS

Minnows (Phoxinus phoxinus L.) were obtained in October from the Freshwater Biological Association, Windermere, some being used without experimentation while others were placed in groups of about 20 in tanks measuring
The temperature was controlled at $18^\circ\pm1^\circ$ C. and, since it was intended also to study the sexual condition of the animals, they were subjected to additional artificial illumination (Bullough, 1940), the intensity of this at the bottom of the tanks being about 130 foot-candles. The results of this illumination have no immediate bearing upon the present report and will not be described here, but it may be mentioned that it was increased by 1 hour per day, starting with 8 hours per day, up to a full 24 hours per day of artificial light. Some of the tanks contained plain tap-water, others a solution of 0.03% thiouracil. The water was well aerated and was changed every 3 days, the fish being fed just previously to this with an ample supply of blowfly larvae supplemented by minced horse-flesh.

Males only were selected for study, and were killed by pithing; the lower jaw and cranium were then fixed separately in mercuric-formaldehyde, the roof of the buccal cavity being first partly removed in order to ensure good fixation of the pituitary without disturbing it. Sections were cut at 5μ in the usual way after wax embedding, and were stained by the Azan method of Heidenhain, the periodic acid Schiff (PAS) method (with or without counterstaining by orange G, Pearse, 1953), and the aldehyde-fuchsin (AF) method of Gomori, as used by Halmi (1952). The significance of these methods in their application to the pituitary of the rat is that the aniline blue of the Azan method reveals the cyanophil cells (the so-called 'basophils' of many earlier authors); the PAS method also stains these cells, and is thus held to indicate them as the probable source of the glycoprotein hormones FSH, LH, and TSH (Pearse, 1952), while the AF method, which stains some, but not all, of the PAS-positive cells has been considered (Purves and Griesbach, 1951) to indicate the thyrotroph cells. It should be made clear, however, that while the chemical basis of the PAS method is well established, the AF method is at present purely empirical (Halmi and Davies, 1953), and the conclusion that the AF-positive cells are thyrotrophs is based upon the observed responses of these cells to thyroidectomy, with some support from a study of conditions in the young animal (Siperstein and others, 1954). We find, as did the latter authors, that the AF method is capricious, and demands careful control of the ripening of the staining solution; we have therefore been careful throughout to check the reaction by staining standard test slides of the pituitary of the minnow and the rat, so that negative results are known to be significant and not to result from inactivity of the reagent, while slides of material from control and goitrogen-treated animals have been taken through the technique side by side.

It is an obvious advantage, when comparing the response of cells to the PAS and AF methods, to be able to stain the same section consecutively by the two techniques. The procedure adopted to this end by Purves and Griesbach in their study of the rat pituitary was to superimpose the PAS coloration upon the AF, the two being distinguished by careful control of the intensity of the staining and of the photomicrographic technique. In the present work it has been found more convenient to bleach the section by
immersion in very dilute 'Milton' after study of the AF reaction, and then to apply the PAS technique. The advantage of this procedure is that it is possible, by examination of the section after the bleaching stage, to establish the complete elimination of the AF coloration, and in this way to guard against the possibility of a false positive response being given in the PAS preparation as a result of the persistence in it of the original AF reaction.

**Observations**

*Normal cytology*

The general structure of the pituitary gland of teleosts, not easily comparable in some respects with that of mammals, has been described and discussed by a number of authors, notably by Kerr (1942) and by Green (1951). It will not, therefore, be dealt with here in detail, and it will be sufficient to say that in the minnow it is composed of two major components which are closely interlocked (fig. 1). The predominantly more ventral of these forms the 'glandular lobe', essentially equivalent to the adenohypophysis of other vertebrates; this is differentiated by its cytological characteristics into three zones, of which the most posterior has been compared with the pars intermedia of the mammalian gland (Green, 1951), the median ('transitional lobe' of some authors) with the pars distalis, and the anterior with the pars tuberalis (Atz, 1953). The second, more dorsal, component may be regarded as the neurohypophysis, and this ramifies by characteristic finger-like outgrowths into the glandular lobe, carrying blood-vessels with it.

Of the various cell-types distinguishable in this gland, the present work is concerned only with those cyanophils which (together with acidophils) are a conspicuous feature of the median zone, and which are also seen, but to a smaller extent, in the anterior zone. The number and size of these cyanophils vary considerably with the seasonal and experimental conditions, the teleost gland being very labile, as Green points out, but in the present material it has been possible to distinguish two main categories. First, there are cells
(type 1, fig. 2, A) with deeply cyanophil contents, the nature of which is not clearly defined in Azan preparations, but which in general have a flocculated or alveolated appearance. The nuclei are often pressed to one side of the cell, stain rather heavily, and may be somewhat flattened, crescent-shaped, or contorted in outline. These cells vary in size, and are undoubtedly enlarged in animals which have been artificially illuminated and in which the reproductive system has been activated. For this reason, and having regard to observations on other fish (see, for example, Kerr, 1948), they are here provisionally regarded as gonadotrophs, the fact that they are PAS-positive (see below) being in accordance with this view.

Contrasting with these is another type (type 2, fig. 2, A), occurring in conspicuous groups in a very characteristic position (fig. 1); as a result of this localization they can readily be distinguished in serial sections, varying in number and position from section to section, but usually lying close against the neurohypophysial tissue, either along the dorso-ventral boundary between the anterior and median zones, or farther back within the latter. These cells, which, for reasons to be explained below, are here regarded as thyrotrophs, are also distinguishable by cytological characteristics. They stain more lightly with aniline blue than do the type 1 cells, and their contents appear more tenuous and even less well-defined. Their nuclei (fig. 2, A) are large and rounded, and in these respects, as in their weaker chromophilia, contrast with those of the other cyanophils. It is not suggested that these cells are only found in conspicuous groups; indeed, individual cells with similar features are certainly to be seen scattered in other parts of the median zone, but their concentration in the manner described is a striking characteristic of them.

The probability that both types of cyanophil are involved in the secretion of glycoprotein hormones is indicated by the fact that they both give a positive reaction in PAS preparations (see above, p. 194), in which they are

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**Fig. 2 (plate).** A to G, pituitary gland of minnow; H and I, thyroid gland of minnow.

A, type 1 cells lie at the lower border, and a type 2 cell is in focus at the top centre, immediately above a clear strip of neurohypophysial tissue. A smaller cyanophil (see text) lies just below the latter to the right of centre. (Azan preparation.)

B, PAS preparation showing type 1 cells below and type 2 cells above; both are PAS-positive.

c, vacuolation of type 2 cells in an animal which had been immersed for 94 days in thiouracil solution. (Azan preparation.)

D, AF preparation, showing the positive response of type 2 cells.

E, PAS preparation of the same field as in D, showing the PAS-positive response of type 2 cells.

F, AF preparation. A strong positive response is shown in the type 2 cells, which extend in a curved line from the top left corner. Type 1 cells, situated centrally, are negative and are only faintly distinguishable.

G, PAS preparation of the same field as in F. Only a very light reaction has been evoked (compare fig. 2, B which illustrates a much stronger one), but the type 1 cells are now clearly indicated in the centre.

H, thyroid gland of control minnow, showing normal follicles.

I, thyroid gland of a minnow which had been immersed for 94 days in thiouracil solution. The type 2 cells of the pituitary of this fish are shown in fig. 2, C.
FIG. 2
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distinguishable from each other by their distribution and nuclear characteristics, the use of the orange G counterstain serving to differentiate both of them from the other cells present. The type 2 cells (fig. 2, B) show a fine magenta-coloured reticulum or granulation, which may form a fringe closely embracing the nucleus. The type 1 cells (fig. 2, B) show a similar coloration, but many of them, usually the larger, are also distinctly acidophilic in appearance as a result of the existence in their cytoplasm of granules or droplets which take up the orange G. Comparison with Azan preparations makes it clear that in these the acidophil inclusions are almost completely unstained, their positions being represented by the clear spaces amongst the blue-stained flocculi or reticulum. They seem to be particularly characteristic of the type 1 cells, but the possibility that they may occasionally be present in type 2 cells has not been entirely excluded.

The use of the AF technique produces an interesting cytological picture in which the nerve fibres of the tractus hypothalamo-hypophyseos, and the reticulum to which they give rise in the neurohypophysis, are stained an intense purple; it would therefore appear that this method provides another means of revealing the presumed neurosecretory material which has recently been effectively demonstrated by chromium haematoxylin (Bargmann and Hild, 1949). In *Astyanax* (Atz, 1953) this method apparently colours all the cyanophils in the median zone, but in the minnow it differentiates the type 2 from the type 1, the contents of the former giving a delicate but well-defined purple coloration while the latter are almost or completely unstained. The close correspondence, amounting to virtual identity, between the AF-positive and PAS-positive material of the type 2 cells is illustrated by a comparison of figs. 2, D and 2, E, which show the same field of these cells stained first by the former technique (fig. 2, D) and then, after bleaching, by the latter (fig. 2, E). If the section is coloured relatively lightly with the AF procedure (using an orange G counterstain), the type 2 cells alone take a distinctive purple colour, the type 1 appearing greyish or greyish-brown by contrast. If a stronger reaction is obtained by prolonging the time of treatment, the purple colour may be taken up slightly by the type 1 cells, but the reaction of the type 2 remains distinctive by its brightness and clarity. It can, therefore, safely be said that this latter type is distinguishable from type 1 by its strongly positive AF reaction, and this difference in the behaviour of the two types may be seen by comparing figs. 2, F and 2, G. The former shows a field coloured by the AF technique, with type 2 cells sharply indicated and type 1 only faintly visible. Fig. 2, G shows the same field after bleaching and re-staining with the PAS technique; the reaction here is a weak one, the type 2 cells being only very lightly coloured, as may be judged by comparing them with the more strongly coloured ones in fig. 2, B, but the type 1 cells are now clearly indicated in complete contrast to their virtually negative AF response.

There is much variation in the size of the cyanophils, dependent upon the physiological condition of the animal, but in particular there are smaller
cells which, in Azan preparations, are not easily assignable to either of the main two types. These often have a nucleus (fig. 2, A) which is contracted and densely chromophil, and may be contorted or deeply incised to such an extent as sometimes to make the cell appear multinucleate. Some of these grade into the type 1 cells, others can be seen to be AF-positive, but it is not clear whether they are all to be regarded as divisible into two types like the larger ones, or whether many of them are undifferentiated cells from which either type could arise. Some at least are probably resting cells from which the larger and active ones are recruited, others may be exhausted ones, and a final opinion on them is reserved until further work on the effect of illumination has been completed.

The effect of thiouracil

Reference has been made above to the view of Purves and Griesbach (1951) that those cells in the rat which are both PAS-positive and AF-positive are thyrotrophs, and that this is also true of the type 2 cells of the minnow is strongly suggested by the results of immersion of the fish in thiouracil solution.

Nine control fish, kept in plain water for 58 days, all had thyroids which were normal in appearance (fig. 2, H), with a low epithelium and with follicles well filled with colloid. In contrast to this, ten fish which had been treated with thiouracil (seven for 59 days and three for 94 days) were highly goitrous, showing enormous enlargement of the thyroid cells with a substantial and sometimes virtually complete loss of colloid (fig. 2, I), indicative, of course, of a heavy outpouring of TSH in response to the inhibitory action of the goitrogen on synthesis of the thyroid hormone.

Now Atz (1953) found that treatment of Astyanax with thiourea produced increased vacuolation and partial degranulation of the cells believed by her to be thyrotrophs, and in the minnow the thiouracil treatment can often be seen to produce a similar effect, although there is variation amongst individuals. Usually, however, it is easy to locate, in the expected areas, groups of type 2 cells, still to some extent cyanophilic in reaction, but showing varying degrees of vacuolation or breaking-down of their contents (fig. 2, C). Their appearance contrasts markedly with the homogeneity of the rest of the gland, which seems to be unaffected by this treatment, and certainly suggests that they may well be thyrotrophs, their condition resulting from their intense secretory activity promoted by the goitrogen treatment. The fact, however, that these cells, unlike the corresponding ones in Astyanax, can be differentiated from the type 1 cells by their AF response makes it possible to carry the analysis further by developing a more detailed comparison with the mammal.

Purves and Griesbach (1951) found that the AF reaction disappeared from the thyrotrophs of the rat after surgical thyroidectomy, a result which they ascribed to the increased output of TSH, the AF reaction being, presumably, in some way an indication of the presence of stored TSH which was lost
from the cells when the demand was increased by the removal of the circulating thyroid hormone. In order to provide a more exact comparison with the present study of the minnow, which has involved chemical and not surgical thyroideectomy, 20 mg. of thiouracil per day was administered orally to rats ranging in weight from 416 g. to 535 g. After 12 days of this treatment there was a marked weakening (although not a complete disappearance) of the AF reaction in the pituitary, accompanied by the expected goitrous reaction of the thyroid. It is thus reasonable to expect a reduction or elimination of the reaction in the AF-positive cells of the thiouracil-treated minnows if they are, in fact, thyrotrophs, and this is exactly what actually occurs, for the nine control animals, like others which had not been maintained in the laboratory at all, showed a clear positive AF reaction, while in the ten thiouracil-treated fish the reaction was very weak or absent.

The slight variation found here may be due in part to variations in the reactivity of the reagent, although since slides from a number of animals were always stained together in groups this is not likely to be very marked. More significant are the undoubted variations in the responses of individual fish to the goitrogen treatment, an extreme example of this being provided by an eleventh thiouracil-treated animal, which, despite having been immersed in the goitrogen for 59 days, differed from the others in having a much more normal thyroid, with abundant colloid and an epithelium only slightly increased in height. The significant feature of this fish was that it also differed from the others in having a clear positive AF reaction in the type 2 cells. Presumably it had proved relatively refractory to the goitrogen, the demand for TSH had remained small, and this was precisely mirrored in the persistence of the AF reaction. Another, although less extreme, example was encountered during the examination of the three fish which had been treated for 94 days with thiouracil. One of these, in contrast to the specimen illustrated in figs. 2, c and 1, showed type 2 cells which, despite some signs of vacuolation, retained a considerable amount of cyanophilia and some of which still gave a very faint positive AF response. The thyroid of this animal proved to retain a marked amount of colloid, despite the pronounced hypertrophy of its cells, so that again there was a correlation between the appearance of this gland and the condition of the type 2 cells, the latter being indicative of a less intense demand for TSH than had been experienced in some of the other fish.

Such facts suggest a very close similarity between conditions in the minnow and in the rat, the positive AF response being, in other words, an index of the presence of stored TSH and of a limited demand for this hormone. Further evidence for this was obtained by transferring some fish to plain water after 59 days of thiouracil treatment, and allowing them 32 days of recovery. At the end of this period three animals were selected at random for comparison with those which had received a full 94 days' treatment with the goitrogen. The latter, as already explained, had a highly goitrous thyroid, but the former showed a restitution of the normal histological appearance of
the gland, with a low epithelium and abundant colloid. Evidently the TSH demand had been greatly reduced in these during the recovery period, and on the view advanced above it would be expected that their type 2 cells might be AF-positive as a result of resumption of TSH storage. This was in fact the result actually obtained, the pituitaries of all three showing a rather slight but quite definite positive reaction.

**DISCUSSION**

The results recorded here are based upon a study of 28 male fish (11 thiouracil-treated, 3 thiouracil-treated with a period of recovery ensuing, and 9 controls, together with 5 which had received no laboratory treatment at all), and are uniformly consistent with the view that two types of cyanophil can be distinguished in the median zone of the glandular lobe of the pituitary, and that TSH secretion is the property of that type which gives a positive AF response and which is found in particular abundance at the boundary between the anterior and median zones, although it is probably not exclusively localized there. The two types can also be distinguished by their nuclear characteristics, and in this respect appear to correspond with the two types described by Atz (1953) in *Astyanax*, in which genus the supposed thyrotrophs have usually a round or oval nucleus, while the supposed gonadotrophs (type 1 of the present account) have nuclei which, while frequently round or oval, may also be sharply indented, bean-shaped, crescent-shaped, or twisted, as is very often the case in *Phoxinus*. Both types are stained by the AF reaction in *Astyanax*, and in this respect, as in their distribution within the gland, the situation in the minnow is different, but a study of a wide range of genera is required before any useful comment can be made on such differences between members of a group as large and as variable as the Teleostei. It has been mentioned above (p. 198) that the smallest cyanophils cannot be as certainly divided into two categories as can the larger ones, and, as Atz (1953) has remarked, the limits of variability of the cell types and the degree of ultimate discontinuity between them are as yet uncertain. Much further work is clearly needed to elucidate the mechanism by which fluctuations in pituitary activity in these fish are effected, and to determine how far one type is recruited at the expense of others.

There has been little reference in studies of the pituitaries of other teleosts to differentiation of the cyanophils. Olivereau (1954) has observed signs of activity in these cells in the salmon at periods of thyroid hyperactivity, and has concluded that they are indicative of TSH secretion, but she does not refer to any specific differentiation of thyrotrophs and gonadotrophs. Kerr (1942), on the other hand, has given purely cytological criteria for the existence of two types of cyanophil in the perch, and it will clearly be of interest to determine whether such cells can be further characterized by the techniques described here. Evidence is also needed as to the source of ACTH, but in the meantime the results of the present work provide a basis for a more critical
analysis of the reactions of the pituitary gland of the minnow to experimental or seasonal conditions than has previously been possible. Only when such an analysis has been made for a particular species does it become possible to determine whether the rate of secretion of the various glycoprotein hormones of the pituitary is linked in any way, or whether they are entirely independent of each other and influenced by independent external factors. This is an issue of importance, having regard to the evidence for the existence of seasonal variation in thyroid activity in teleosts (Barrington and Matty, 1954), and it is hoped to present later some observations on this aspect of the problem.

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REFERENCES

KERR, T., 1948. Ibid., 89, 129.