A HISTOCHEMICAL STUDY OF THE ESTERASES OF RAT THYROID AND THEIR BEHAVIOUR UNDER EXPERIMENTAL CONDITIONS

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IT has been known for a long time that changes in thyroid activity are accompanied by definite cytological changes in the gland, but little is known about the associated enzyme changes or their significance. A proteolytic enzyme has been found by de Robertis (1949) in the colloid of active thyroid follicles, and Schachner, Franklin and Chaikoff (1943) obtained evidence that the cytochrome–cytochrome oxidase system was involved in secretory activity. Okkels (1934) has related the distribution of oxidase granules in the thyroid of the guinea-pig injected with thyrotrophic hormone to the functional state of the gland and peroxidase activity was demonstrated in the cells of the thyroid follicles by Dempsey (1944), Glock (1944), and de Robertis and Grasso (1946).

Alkaline glycerophosphatase was demonstrated in the capillaries of the mammalian thyroid by Gomori (1941) and by Kabat and Furth (1941). Wolf, Kabat and Newman (1943) mention the presence of acid phosphatase in the nuclei and cytoplasm of the thyroid epithelium but give no details as to its localization in the cell. Dempsey and Singer (1946) found alkaline glycerophosphatase in the follicular epithelium of peripheral inactive follicles in the rat thyroid and acid glycerophosphatase in the cells of the central active follicles. They also demonstrated glucose phosphatase and fructose diphosphatase in the capillary endothelium.

With regard to the carboxylic acid esterases, Gomori (1946), using Tween 40 or 60 and Product 81 as substrates, found no lipase in the thyroid of the rat or of any of the other species which he examined. Huggins and Moulton (1948) quantitatively demonstrated the presence of a non-specific esterase in the rat thyroid with *p*-nitrophenyl propionate as substrate and Weber (1954), using α-naphthyl acetate and the Tweens, studied the enzymes of rat thyroid after treatment with thyroxine and thyrotrophic hormone. He found an enzyme activated by sodium taurocholate and not inhibited by 0·001 M eserine and suggested that it was an aliesterase, probably a lipase. Dejardin (1955) described the distribution of pseudocholinesterase in guinea-pig thyroid. He found this enzyme to be present in so-called replacement or chromophile cells.

The present report deals with the nature and distribution of rat thyroid esterases using *o*-acetyl-5-bromoindoxyl (5. Br. I) and acetylthiocholine (AThCh) in association with various inhibitors. The two enzymes which were identified have been studied under various experimental conditions.

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MATERIAL AND METHODS

Rats.—Male albino rats weighing approximately 200 g. were used.

Tissues.—The rats were killed with coal gas and a block of tissue containing the thyroid attached to the trachea was immediately removed and fixed in cold (4°) 15 per cent formalin containing 1 per cent $CaCl_2$ (pH 7·4) for 24 hr. Sections were cut at 15 μ on a freezing microtome, washed in distilled water for a few minutes and then incubated in the substrate mixtures. In order to counteract the error arising from variations in thickness, 8–10 sections from each gland were taken in almost unbroken succession and placed in the individual incubation mixtures.

Substrates.—1. *o*-Acetyl-5-bromoindoxyl. This was used according to the method described by Pearse (1953) except that the pH used was 6·2.

2. Acetylthiocholine. Gomori's (1952) modification of the Koelle method was used.

Incubation (37°).—With the indoxyl substrate the sections were incubated for 45 min. With acetylthiocholine as substrate the optimum time was found to be 8 hr.

Inhibitors.—The following inhibitors were used for the identification of the enzymes :

62C47 (1 : 5-bis-(4-trimethylammonium-phenyl)pentan-3-one diiodide, 0·001 M ; *iso*-OMPA (tetra-*iso*propyl pyrophosphoramide), 0·001 M ; E600 (diethyl-*p*-nitrophenyl phosphate 0·0001 M ; silver nitrate, 0·02 M ; sodium taurocholate, 0·05 M ; sodium dodecyl sulphate, 0·01 M ; hydrocinnamic acid, 0·01 M ; hydroxylamine, 0·01 M ; sodium fluoride, 0·01 M.

When the inhibitors were used the sections were first incubated in solutions of these for 1 hr. and then transferred to the substrate mixture containing the same concentration of inhibitor, except in the case of the irreversible inhibitors.

Fat stains.—In order to correlate enzyme changes with fat metabolism the following stains were employed :

For neutral fats : Oil Red O, Sudan III and IV and Phosphine 3R. For phospholipids : Sudan Black B and Baker's acid haematein method.

Experiments

For this purpose a total of 26 rats was used, divided into the following groups :

Group I.—Daily subcutaneous injections of 1 U.S.P. unit thyroid stimulating hormone (T.S.H.) were given to 3 rats. The hormone preparation used was Armour's Thytropar freshly dissolved in saline before use. These rats were killed on the 2nd, 4th and 8th days after starting the injections.

Group II.—A saturated solution of 4-methyl-2-thiouracil in place of the ordinary drinking water was given to 10 rats, of which one was killed on alternate days from the 4th to the 22nd day inclusive.

Group III.—Daily injections of 3 μg./100 g. rat of L-thyroxine subcutaneously were given to 10 rats in drinking the saturated solution of 4-methyl-2-thiouracil. The thyroxine was made up daily as follows : 1 tablet of sodium L-thyroxine (0·1 mg.) was dissolved in 50 ml. of normal saline to which had been added 40 mg. sodium carbonate. These rats were killed at the same times as those of group II.

Group IV.—Auto-implants of half of the thyroid gland were done on a group of 3 rats according to the method described by Dempster and Doniach (1955). These were killed on the 9th day after the operation. Control animals were killed with all the experimental animals.

RESULTS

Histochemistry of the Thyroid Esterases

Indoxyl esterase.—The enzyme demonstrated by using *o*-acetyl-5-bromoindoxyl is present in all the acini throughout the gland, but in slightly higher concentration in the peripheral and isthmic acini than in those in the centre of the thyroid lobes. It is present throughout the cytoplasm of the individual cells, but usually shows a higher concentration near the luminal border of the cell (Fig. 1). In some acini this appearance is so distinct that the precipitate of 5-5′-dibromoindigo forms an almost complete circle between the nuclei and the lining

of the follicle. No enzyme could be detected in the nuclei and in the normal gland the colloid was completely devoid of enzyme activity.

The reaction of this enzyme towards the various inhibitors and activators is given in the Table.

TABLE.—*Effect of Inhibitors and Activators on Thyroid Indoxyl Esterase*

Inhibitor	Per cent activation	Per cent inhibition
Sodium fluoride 0·01 M	—	—
Hydroxylamine 0·01 M	—	—
$AgNO_3$ 0·02 M	—	100
Cysteine 0·01 M	30	—
Hydrocinnamic acid 0·01 M	—	—
Sodium dodecyl sulphate 0·01 M	—	30
E600 0·0001 M	—	100
Sodium taurocholate 0·05–0·01 M	30	—
62C47 0·001 M	—	—
iso-OMPA 0·001 M	—	—

Because both 62C47 and *iso*-OMPA had no effect on the indoxyl esterase it cannot be a cholinesterase, either specific or non-specific. Inhibition by E600 confirms its general identity as a carboxylic acid esterase and inhibition by sodium dodecyl sulphate suggests that it may be a lipase. Cysteine activation and silver nitrate inhibition characterize those esterases which probably act *in vivo* as peptidases, and stimulation by sodium taurocholate, though often considered to favour lipase rather than simple esterase, we have found to characterize peptidase-type esterases elsewhere in the body (Pepler and Pearse, 1956).

Cholinesterase.—The enzyme in the thyroid parenchyma was completely inhibited by 0·001 M *iso*-OMPA and not at all affected by 62C47 at the same concentration. This indicates that it is a non-specific or pseudocholinesterase. Acetylcholinesterase was limited to the plexuses of fine cholinergic fibres surrounding the follicles.

The pseudocholinesterase always had a patchy distribution in that the centrally placed follicles regularly contained small amounts of the enzyme whereas peripherally the majority of follicles showed no enzyme activity. However, occasional follicles at the periphery of the lobes and in the isthmic portion, although lined by flat cuboidal epithelium, contained a large amount of the enzyme (Fig. 2). As a rule the enzyme was evenly distributed in the cells of individual follicles, but occasionally only some of the cells showed enzyme activity. Frequently the enzyme was localized in circular bands or dispersed in small groups of cells without any regularity. In a given cell the enzyme appeared to be concentrated in two sites, around the nucleus and near the luminal border. In the occasional follicle with a very intense enzyme reaction in the cells, slight staining was also observed in the colloid, but otherwise no enzyme could be demonstrated here. The nuclei were always free of enzyme activity.

Experimental Results

Group I (TSH).—In the rat killed on the 2nd day of the experiment the amounts of both the indoxyl and pseudocholinesterase appeared to be within normal limits. However, the rats sacrificed on the 4th and 8th days showed a definite decrease of the indoxyl esterase (Fig. 3). The pseudocholinesterase

showed a decrease per cell and per lobule as a whole, but in areas with strong enzyme activity the reaction still remained very intense.

Group II (*Thiouracil*).—The rat killed 4 days after the beginning of the experiment showed a distinct decrease of the indoxyl esterase in the central reactive part of the lobe. In addition there also appeared to be a redistribution of the enzyme in the cells so that, although it was still present throughout the individual cells, there was a relatively heavier concentration near the luminal border. At 8 days the decrease in enzyme activity was apparent in the whole gland. At 12 days the basal parts of the cells in some follicles were entirely free from enzyme and the luminal part of the cell showed only faint activity (Fig. 4). Some of the follicles, especially in the central part of the lobe, were almost devoid of enzyme activity. In the rats killed at later stages the overall picture remained stationary although the cells were even more hypertrophic.

Owing to the patchy distribution of the pseudocholinesterase, it was difficult to assess early changes in the amounts of the enzyme with any accuracy. In the rats killed on the 8th day however, it became clear that there was a decrease in this enzyme. In the later stages of the experiment, that is, in the rats killed from the 16th–22nd day, only occasional cells were found in the 8 sections examined which showed any residual enzyme activity.

Group III (*Thyroxine–thiouracil*).—In the majority of rats the dosage of thyroxine employed seemed adequate to prevent stimulation of the thyroid cells by thyrotrophic hormone and in these the amounts of both the indoxyl and pseudocholinesterase showed no significant variation from that of the control series.

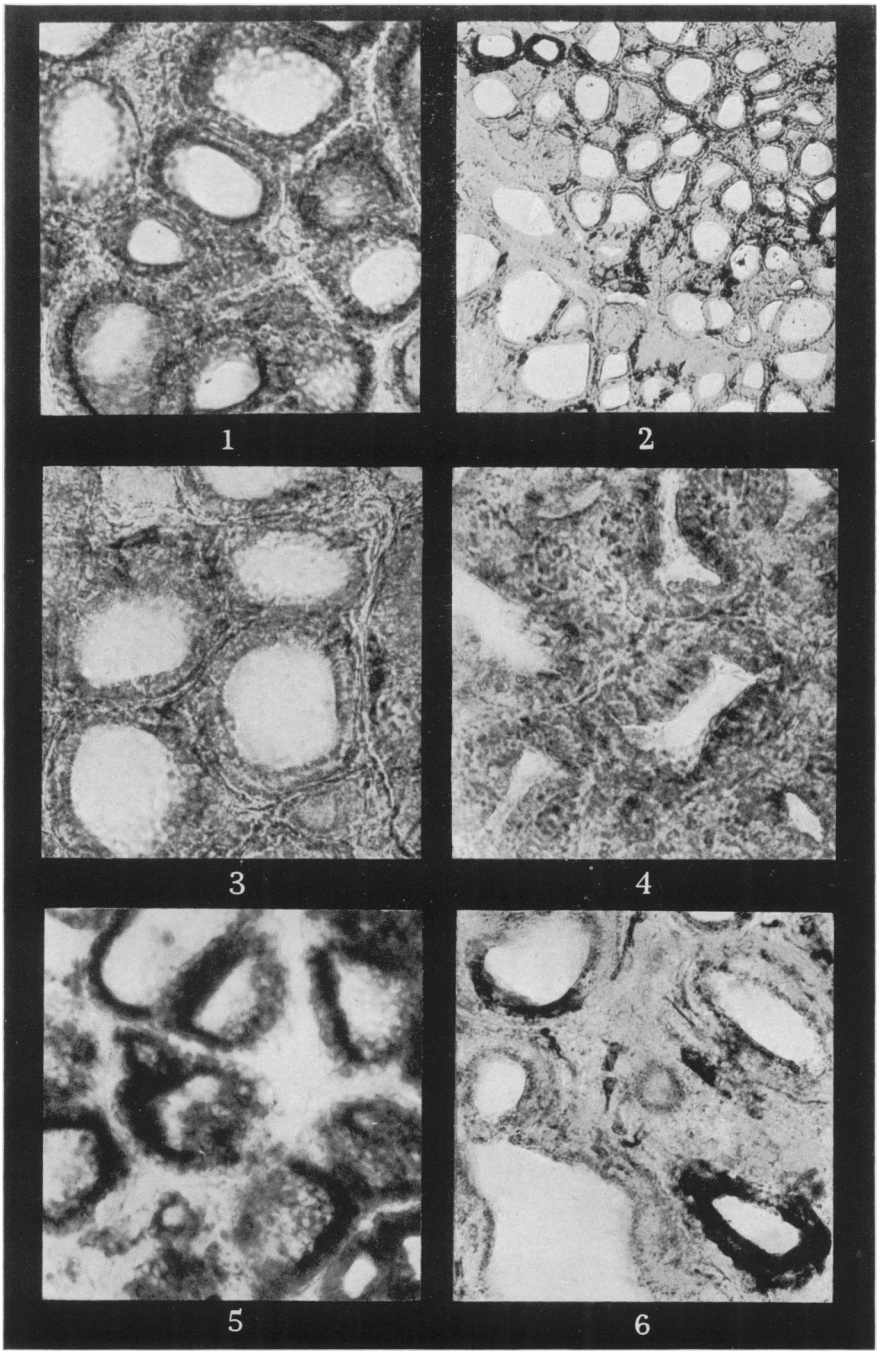
Group IV.—As shown in Fig. 5 and 6, the indoxyl and pseudocholinesterases could still be demonstrated in the auto-implants removed on the 8th day after the operation.

DISCUSSION

Although the presence of a non-specific esterase in the thyroid is fairly well established (Huggins and Moulton, 1948), there is still considerable controversy with regard to the presence or absence of a lipase (cf. Gomori, 1946; Weber, 1954). After inhibition of the pseudocholinesterase (Table I), the reaction of our indoxyl esterase towards $AgNO_3$, cysteine and E600, suggested that the possibility of its being a lipase could not be ruled out altogether, especially in view of the fact that it was stimulated by taurocholate. In order to pursue this matter further we decided to correlate variations of the enzyme under experimental conditions with the lipid metabolism of the gland. In this respect further difficulties were encountered because although Dempsey (1949) and Hassler, Gislen and Brolin (1953) were able to demonstrate lipids in the epithelial cells, the former claimed an

EXPLANATION OF PLATES

FIG. 1.—Indoxyl esterase in rat thyroid (control). × 190.
FIG. 2.—Pseudocholinesterase in rat thyroid (control). Note the patchy distribution of the enzyme in the follicles. × 50.
FIG. 3.—Indoxyl esterase in rat thyroid (T.S.H. for 8 days). × 190.
FIG. 4.—Indoxyl esterase in rat thyroid (thiouracil for 12 days). × 190.
FIG. 5.—Indoxyl esterase in auto-implant of thyroid removed after 9 days. × 205.
FIG. 6.—Pseudocholinesterase in thyroid auto-implant. × 205.



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increase of cytoplasmic lipids under conditions of stimulation of the thyroid, whereas the latter stated that the amount of lipid was decreased. It therefore seemed necessary to repeat this work in the present series of experiments. We were, however, unable to demonstrate any significant amount of neutral fat or phospholipid with the methods used in either the control rats or the rats stimulated with thiouracil. In an occasional section stained with Oil Red O there was a suggestion of a slight pink staining near the luminal border of the cells, but this was by no means a constant finding. We believe, therefore, that there is no correlation between the indoxyl esterase and either phospholipid or neutral fat metabolism.

Recent work (Kirschner, 1953; Koch, 1954) has indicated that the cholinesterases may be involved in the active transport of ions (Na^+ and Cl^-) through cell membranes. This led us to investigate the possibility that the pseudocholinesterase in the thyroid might have a similar function in relation to the metabolism of iodide. It is well known that both acetyl- and pseudocholinesterase are present in peripheral nerves (Cavanagh and Webster, 1955; Cavanagh, Thompson and Webster, 1954) and it was therefore necessary to make sure that the pseudocholinesterase was not associated in any way with the nerve supply of the gland. In order to prove this, auto-implants of thyroid were done and the implants were examined after sufficient time had elapsed for the nerves to degenerate. By this means it was possible to show that the enzyme belonged to the thyroid cells proper and was not associated with the nerve supply. The findings of Morton, Perlman and Chaikoff (1941) that thyrotrophic hormone increases assimilation of iodine by the thyroid gland suggested to us that the action of this hormone might be explained on the basis of an increase in enzymes concerned with the trapping of iodine. In view of the work of Kirschner (1953) and Koch (1954) it was thought that the enzyme might be a cholinesterase. The effect of T.S.H. on the pseudocholinesterase was forthwith tested but, if anything, it caused a decrease in the amount of enzyme in the gland rather than an increase. This decrease, however, might have been unassociated with any direct action of the hormone on the enzyme system and could have been due to the associated hypertrophy of the cells. In order to check these results a second group of rats was given thiouracil. The resultant hypertrophy of the thyroid in this group, produced by endogenous thyrotrophic hormone stimulation, was again associated with a definite decrease in the pseudocholinesterase content of the gland. Although it seemed unlikely that thiouracil would affect this enzyme directly, we thought it necessary to control this. Thyroxine was therefore given to a third group of rats (in addition to the thiouracil) in doses that would suppress endogenous pituitary thyrotrophic hormone production (Griesbach, Kennedy and Purves, 1949; Purves and Griesbach, 1946). The results obtained confirmed the fact that the blocking effect of thiouracil was not related to these enzyme systems.

The indoxyl esterase behaved similarly to the pseudocholinesterase under the experimental conditions studied and in this respect our results differ from those of Weber (1954) who found an increase of the α-naphthyl esterase under conditions of stimulation of the thyroid.

SUMMARY

The nature and distribution of enzymes which hydrolyse *o*-acetyl-5-bromoindoxyl and acetylthiocholine in the rat thyroid have been investigated.

On the basis of inhibition studies we suggest that the indoxyl esterase may be a peptidase. The enzyme hydrolysing acetylthiocholine is a pseudocholinesterase.

Attempts to correlate the level of pseudocholinesterase with the level of iodine metabolism were unsuccessful.

The decrease of pseudocholinesterase and indoxyl esterase (peptidase) produced by stimulation of the gland must be regarded as relative rather than absolute, due to hypertrophy of the cells.

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