The Absence of Thyroid Hormones in a Growth Factor of Duodenal Powder

C. J. ACKERMAN
Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia

ABSTRACT Three fractions prepared from the acid-insoluble residue after acid hydrolysis of duodenal powder were effective in reinstating growth of growth-arrested, sulfaguanidine-fed rats. One fraction was studied more extensively than the others to determine whether the growth response to this fraction was due to thyroxine or triiodothyronine. The stability of this fraction to acid hydrolysis, its insolubility in acid, its behavior on paper chromatograms and its failure to react with ninhydrin contrasted with the behavior of thyroxine or triiodothyronine. It was therefore concluded that duodenal powder contains an unidentified substance effective in reinstating growth of growth-arrested, goitrogen-fed rats.

Previous reports (1, 2) have demonstrated that dietary duodenal powder is effective in reinstating growth of growth-arrested, goitrogen-fed rats. A water-soluble fraction prepared from enzymatically hydrolyzed duodenal powder reinstated growth of growth-arrested thyroidectomized, hypophysectomized, sulfaguanidine-fed or thiouracil-fed rats (3). Although the data suggested that the observed growth responses were not due to thyroid hormones, unequivocal evidence for the presence or absence of thyroid hormones in this fraction was needed.

The present paper reports evidence that the growth response of growth-arrested sulfaguanidine-fed rats produced by a fraction obtained from duodenal powder is not due to thyroxine or triiodothyronine. In addition, certain observations suggest that more than one growth factor is present in duodenal powder.

EXPERIMENTAL

Materials and methods. "Active" duodenal powder, "inactive" duodenal powder, and thyroid powder (3 × USP) were obtained from commercial sources. The "active" material was the same material as that used in earlier experiments (2) and when fed at a level of 4 g/100 g of diet, the 2-week growth response of growth-arrested, sulfaguanidine-fed rats was 39 to 49 g. Another preparation of duodenal powder which had no ability to reinstate growth was defined as "inactive."

When tested under the same conditions as the active material, the 2-week growth response to the inactive powder was – 1 to + 1 g/2 weeks. This material was used as a diluent of thyroid powder and of triiodothyronine to provide the same experimental conditions when the stability of thyroid hormones was being compared with that of the growth factor of the "active" duodenal powder. L-Thyroxine (T₄) and 3, 5, 3'-triiodothyronine (T₃) were recrystallized from hot HCl (4, 5); mp 231 to 233° and 202 to 204°, respectively. These were dried over H₂SO₄. All solvents were reagent or C.P. grade, but solvents used for paper chromatography were distilled once. Acidified methanol: 95 ml of methanol plus 5 ml of 2 N HCl; acidified acetone: 95 ml of acetone plus 5 ml of 2 N HCl.

Paper chromatography was conducted in the dark in an all-glass chromatography jar at 5° (descending) unless otherwise specified. Whatman 3 MM paper was used throughout these experiments. Three solvents were used: solvent 1, 0.1 N HCl; solvent 2, methanol:0.2 M ammonium acetate adjusted to pH 4.5 with acetic acid (1:1); solvent 3, acetone:0.2 M ammonium acetate adjusted to pH 4.4 with...
acetic acid (4:6). The fractions obtained from duodenal powder were streaked evenly in a band from edge to edge because the nature of these fractions was such as to retard the solvent flow. When the samples were applied as spots or in bands shorter than the width of the paper, the solvents tended to encircle, or by-pass the samples.

Iodinated compounds on the chromatograms were detected by the ceric sulfate-arsenious acid technique of Kono et al (6). Growth-arrested, sulfaguanidine-fed male rats of the Sprague-Dawley strain (2) were used for the growth assays. Such animals may vary in body weight from 100 to 160 g, but their body weight gain is negligible after the fifth week of goitrogen feeding (2). For example, 50 rats selected at random from 600 rats so treated, gained an average of + 0.34 g ± 2.98 (sd) in 2 weeks. The maximal gain of any rat in this group was + 5 g/2 weeks (one rat). Therefore it was feasible to use one rat per assay to assess qualitatively the growth activity of the various fractions. Unless otherwise specified, the sample to be assayed (usually a methanolic solution) was mixed into 15 to 20 g of the diet containing sulfguanidine (2), air-dried for 3 to 5 hours, and then fed ad libitum to one rat until it was consumed. Feeding of the usual sulfaguanidine-containing diet was then resumed, and the body weight gain at the end of 14 days was used to assess the potency of the growth factor in the sample tested.

Stability to HCl and NaOH hydrolysis. For these experiments, a definite quantity of the material to be assayed was fed to one rat in 100 g of diet. This was consumed in 11 to 12 days. The usual sulfaguanidine-containing diet was then fed for the remainder of the 14-day period. In this way, the effect of the growth factor in duodenal powder could be directly compared with that of a definite quantity of thyroid powder or T3. Also, the recovery of the growth factor or thyroid hormones after acid hydrolysis could be evaluated by comparing the growth responses to the hydrolyzed samples with the growth responses to the samples before hydrolysis. Active duodenal powder (15 g) mixed into 288 g of the sulfaguanidine diet was assayed with 3 rats. Twenty-four grams of active duodenal powder were refluxed for 22 hours with 100 ml of 5 N HCl. The cooled hydrolysate was filtered and the insoluble residue was washed twice with 10 ml of 1 N HCl. One-half of this residue mixed into 300 g of diet was assayed with 3 rats. The other half of the insoluble residue was refluxed with 8 ml of 2 N NaOH for 16 hours. The entire hydrolysate was neutralized with HCl, mixed into 300 g of diet and assayed with 3 rats.

Three levels of thyroid powder, 135, 270, and 405 mg were each diluted to 36 g with "inactive" duodenal powder. These were then treated in exactly the same way as the active duodenal powder, namely, 12 g of the mixture were assayed and 24 g were hydrolyzed with HCl as described above. Hydrolysis with NaOH was omitted. Thus, each rat consumed 100 g of diet containing 15, 30 or 45 mg of thyroid powder or the acid-insoluble fraction obtained from these quantities of thyroid powder. In addition to the above, 315 mg of thyroid powder in 24 g of "inactive" duodenal powder were hydrolyzed with HCl, washed and filtered as described above. One-half of the insoluble residue in 300 g of diet was assayed with 3 rats. Also, 30 μg of T3 were mixed with 36 g of inactive duodenal powder. Twelve grams in 288 g of diet were assayed with 3 rats (3.3 μg of T3/rat). Twenty-four grams were hydrolyzed with HCl, filtered, and one-half of the insoluble residue was assayed as described above.

Preparation of the growth factors. Four hundred grams of active duodenal powder were refluxed for 22 hours in 3000 ml of 5 N HCl. The cooled hydrolysate was filtered through paper and the acid-insoluble residue was air-dried for 16 hours and then extracted with acetone in Soxhlet extractors for 6 hours. An equal volume of benzene was added to the acetone extract. This mixture separated into 2 phases with a black precipitate at the interface. After separating the precipitate and the lower phase from the benzene (upper) phase, the benzene phase was washed 3 times with 30 ml of 10% aqueous acetone and then discarded. All the washings and the insoluble material were combined with the lower
phase. This was evaporated to a black viscous oil at reduced pressure and then dissolved by adding 2 N NaOH until the pH was approximately 12. It was diluted to 2 liters with water and then filtered through paper. The insoluble material was discarded. The filtrate was acidified to pH 1 with HCl, cooled to room temperature and then filtered through paper. The acid-insoluble residue was again dissolved in NaOH and the solution was acidified to pH 5 with acetic acid. After filtering, the precipitate was air-dried for 16 hours and then extracted in a Soxhlet extractor with 95% ethanol for 6 hours. The amber alcohol extract was evaporated to dryness. The residue was dissolved in 100 ml of 0.1 N NaOH, which was then acidified to pH 10 with acetic acid, cooled overnight and filtered. The filtrate was acidified to pH 8 with acetic acid, cooled overnight and filtered. The pH 8 precipitate was dissolved in 100 ml of acidified methanol (fraction 1), and 2 ml were assayed with one rat.

After fraction 1 had been allowed to stand for one week at room temperature in a well lighted room, evaporation of the methanol left a residue which was no longer completely soluble in NaOH. Therefore, the alkaline solution was acidified to pH 4 with acetic acid and extracted with an equal volume of benzene, and 3 times with one-half volumes of benzene. One forty-ninth of this solution was assayed with one rat. Also, another aliquot in 10 ml of methanol:acetate buffer, pH 5 (1:1) was heated for 10 minutes in a boiling water bath with 5 mg of ninhydrin. This was assayed with one rat.

After the pH 8 precipitate (fraction 1) was removed by filtration, the filtrate was adjusted to pH 6 with acetic acid, cooled overnight at 5° and then filtered. The red precipitate was extracted for 6 hours with methanol in a Soxhlet extractor. This was fraction 2 in a volume of 130 ml. When air-dried, 1 ml of this solution contained 14 mg of an oily black residue. The final steps of the isolation procedure, beginning with the pH 5 precipitate, are illustrated in figure 1. Also shown on figure 1 is the subsequent division of fraction 2 into 2 active fractions, which was observed after the tests described below were conducted on fraction 2.

Three milliliters of the solution of fraction 2 were effective in reinstating growth of one growth-arrested rat. Therefore, multiples of 3-ml aliquots of fraction 2 were treated as follows and then assayed with the appropriate number of rats so that the effect of the treatment could be evaluated by comparing the growth responses before and after treatment:

(a) Two 3-ml aliquots were evaporated to dryness in test tubes to which were added 2 ml of 2 N NaOH and 1 mg of thioracil as an antioxidant (7). The tubes were evacuated with a water aspirator and filled with nitrogen 3 times. The tubes were sealed and heated in a boiling water bath for 16 hours. The hydrolysates were neutralized with HCl and then assayed with 2 rats. This entire procedure was

Fig. 1 A diagram of the preparation of Fractions 1 and 2 from the pH 5 precipitate which was obtained from the acid-insoluble residue of acid-hydrolyzed duodenal powder. Further treatment of Fractions 1 and 2 resulted in 3 fractions (heavy underline) which were effective in reinstating growth of growth-arrested, sulfaguanidine-fed rats.
repeated except that the evacuation and filling of the tubes with nitrogen were omitted.

(b) Six milliliters were diluted to 12 ml with methanol to which were added 50 mg of palladium black. This was shaken for 7 hours at room temperature with hydrogen at a pressure of 2.8 kg/cm². The methanol was decanted and the palladium was washed 3 times with 5 ml of acidified methanol. The methanol extracts were combined and assayed with 2 rats.

(c) The growth response to 3 ml of fraction 2 was comparable to that produced by 5 μg of T₄ or 3.3 μg of T₃. These quantities are readily soluble in dilute HCl (8) and washing fraction 2 with HCl may be expected to decrease its activity if these hormones are present. Therefore, 9 ml of fraction 2 were evaporated to dryness and the residue dissolved in 9 ml of 0.1 N KOH. To this was added 1 ml of 12 N HCl. The precipitate was redissolved in KOH and precipitated with acid 2 times, and the last HCl solution, containing the precipitate, was extracted once with an equal volume of benzene. The yellow benzene and the acid extracts were discarded. The precipitate, dissolved in methanol, was assayed with 3 rats.

(d) If present in fraction 2, thyroid hormones could be bound in a form resistant to acid hydrolysis. To test this possibility and demonstrate the solubility of thyroxine in HCl, 6 ml of fraction 2, and 6 ml of fraction 2 to which had been added 10 μg of T₄, were evaporated to dryness in separate test tubes. These were hydrolyzed in 4 ml of 2 N NaOH as described in experiment (a) above. The alkaline hydrolysates were acidified to pH 1 with HCl and centrifuged. The precipitates were dissolved in 9 ml of 0.1 N NaOH and reprecipitated with HCl. This was repeated once. The combined HCl extracts and the insoluble precipitates were assayed with 2 rats for each fraction.

(e) Two 3-ml aliquots were each applied onto 2 large sheets of filter paper (56 × 47 cm) in bands 56-cm long (0.05 ml of sample/cm). On 2 separate strips 2-cm wide, 2 μg of T₄ and 2 μg of T₃ in 0.11 ml of fraction 2 were applied in bands 2-cm wide. The chromatograms were developed with solvent 1 at room temperature for 16 hours. The T₄ and T₃ were located on the narrow strips with ceric sulfate-arsenious acid.

The narrow orange bands at the origin of the large chromatograms were cut off, eluted with methanol in Soxhlet extractors for 3 hours and the methanol extracts were assayed with 2 rats. The remainder of the chromatograms (R₉ 0.03 to 1.0) were also eluted with methanol and the extracts were assayed with 2 rats. This procedure was repeated except that 10 μg of T₄ were co-chromatographed with 6 ml of fraction 2 to demonstrate that the growth factor could be separated from thyroid hormones in this manner.

(f) Experiment (e) was repeated except that the chromatograms were developed with solvent 2 and then divided into 4 sections each of which were eluted with acidified methanol. The extracts were assayed with 2 rats each.

(g) Three milliliters on filter paper in a band 32-cm wide were developed with solvent 3. Three sections of the chromatogram were extracted and each extract was assayed with one rat.

(h) Three milliliters in a band 32-cm wide on filter paper were developed with solvent 3. The section 0.85 to 1.0 was sprayed on both sides with 0.3% ninhydrin in acetone. When the paper had dried, it was again sprayed on both sides with ninhydrin and then allowed to stand 20 hours at room temperature. The section 0.85 to 1.0 was cut off, eluted with acidified methanol, and the extract was assayed with one rat.

(i) The residue from 3 ml was dissolved in methanol:0.1 N acetate buffer, pH 5 (1:1). To this were added 5 mg of ninhydrin which was heated for 10 minutes in a boiling water bath. The red solution was assayed with one rat.

(j) Fifteen micrograms of T₄ on filter paper (10 × 5 cm) were treated with ninhydrin as described above (exp. (h)). The paper was extracted with acidified methanol which was then assayed with one rat. Also, 400 μg of T₄ treated with ninhydrin as described in experiment (i) above, were assayed with 2 rats.

(k) After drying 15 ml of fraction 2 on filter paper (10 × 15 cm), the paper was

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8 Unpublished experiments.
extracted for 3 hours with benzene in a Soxhlet extractors. The benzene was removed and when the paper was air-dried, it was extracted with acetone for 3 hours. This was followed by a 3-hour extraction with acidified acetone and finally by a 3-hour extraction with methanol. Two-fifths of each extract were assayed with 2 rats each. The preparation of these fractions is illustrated in figure 1.

RESULTS
Because of the limited number of rats used in the experiment summarized in table 1, it was not intended to quantitatively assess the growth response to duodenal powder, thyroid powder or T3. However, the results show that 4 g of duodenal powder produced a growth response approximately equivalent to that produced by 15 mg of thyroid powder (3 × USP) or 3.3 μg of T3. If duodenal powder contained thyroid hormones in amounts 3 times that amount present in 15 mg of thyroid powder, they would not be recovered in the acid-insoluble residue after acid hydrolysis. The acid-insoluble residue from 45 mg of thyroid powder produced little, if any, growth response, whereas the acid-insoluble residue from 4 g of duodenal powder produced a growth response almost equivalent to that of the untreated duodenal powder. The acid-insoluble residue from the higher level of thyroid powder (52 mg) did retain some ability to reinstate growth but triiodothyronine at the level tested, was not recovered after acid hydrolysis.

The growth factor in the acid-insoluble residue obtained from active duodenal powder was partially destroyed by NaOH hydrolysis. The growth response of 3 rats to the alkaline hydrolysate (see Experimental section) was 15, 19 and 22 g/2 weeks. Since the acid-insoluble residues obtained from thyroid powder were inactive, NaOH hydrolysis of these fractions was omitted.

After acid hydrolysis of active duodenal powder, 2 fractions effective in reinstating growth were recovered from the acid-insoluble residue. Table 2 summarizes the growth response to these fractions before and after various treatments. Fraction 1 (precipitated at pH 8) became soluble in benzene (fig. 1) and this fraction lost its ability to reinstate growth after it had been treated with ninhydrin. Further work on this fraction will not be reported here. Emphasis was placed on fraction 2 (the pH 6 precipitate) to determine whether the growth response produced by this fraction was caused by thyroid hormones.

The instability of the growth factor to alkaline hydrolysis suggested a method for distinguishing the growth factor from the thyroid hormones which are relatively stable to alkaline hydrolysis under the proper conditions. However, fraction 2 was stable to alkaline hydrolysis when air was excluded although it lost all activity when air was present (table 2, (a)). This, and its instability to catalytic reduction (table 2, (b)) did not distinguish the growth factor from the thyroid hormones.

TABLE 1

<table>
<thead>
<tr>
<th>Sample fed/rat</th>
<th>Results</th>
<th>HCl-hydrolyzed acid-insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td></td>
</tr>
<tr>
<td>4 g duodenum</td>
<td>39, 40, 45</td>
<td>32, 36, 38</td>
</tr>
<tr>
<td>15 mg Thyroid powder</td>
<td>40, 44, 49</td>
<td>0, 2, 3</td>
</tr>
<tr>
<td>30 mg Thyroid powder</td>
<td>48, 48, 51</td>
<td>2, 2, 5</td>
</tr>
<tr>
<td>45 mg Thyroid powder</td>
<td>44, 53, 59</td>
<td>6, 8, 10</td>
</tr>
<tr>
<td>52 mg Thyroid powder</td>
<td>—</td>
<td>15, 23, 25</td>
</tr>
<tr>
<td>3.3 μg Triiodothyronine</td>
<td>36, 44, 47</td>
<td>0, 3, 4</td>
</tr>
</tbody>
</table>

1 No rat gained more than 4 g in the 2 weeks prior to these experiments.
2 Each value represents the body weight gain of one rat that had been fed the indicated sample in 100 g of diet.
3 The acid-insoluble residue after duodenal powder, or thyroid powder mixed with "inactive" duodenal powder, was boiled for 23 hours with 5 w HCl.
4 Thyroid powder and triiodothyronine diluted with an inactive preparation of duodenal powder, were mixed into the sulfaguanidine-containing diet. This was consumed in 11 to 12 days.
However, little growth activity was lost after extraction of fraction 2 with HCl and benzene (table 2, (c)) and it was concluded that neither T₄ nor T₃ was present in a free form. That neither T₄ nor T₃ were present in a bound form was demonstrated when alkaline hydrolysis, and washing with acid failed to decrease the growth activity of the acid-insoluble residue (table 2, (d)). Thyroxine added to this fraction before alkaline hydrolysis was recovered in the acid-soluble extract, demonstrating that it would have been removed from fraction 2 if it had been present.

That the growth factor differs from T₄ and T₃ was also demonstrated by paper chromatography. After 16 hours’ development with solvent 1, the solvent front had moved off the end of the paper, but T₄ and T₃ had moved 20 and 33 cm, respectively, from the origin (fig. 2). However, the R₁ of the growth factor was zero, and 10 μg of T₄, co-chromatographed with fraction 2 were recovered in the area R₁ 0.03 to 1.0 (table 2, (e)).

With solvent 2, the R₂ of the growth factor was again zero and T₃ added to fraction 2 was recovered in R₂ section 0.3 to 0.65 (fig. 2 and table 2, (f)).

When fraction 2 was developed on paper chromatograms with solvent 3, an orange band moved with the solvent front, and this contained all of the growth activity of the sample (table 2, (g)). The R₂’s of T₃ and T₄ with this solvent were 0.80 and 0.85, respectively, but when another chromatogram developed with this solvent was sprayed with ninhydrin, the growth response (table 2, (h)) indicated that the growth factor was neither T₃ nor T₄, and the growth factor probably contains no free amino group. The reaction with ninhydrin was tested again by treating fraction 2 in solution with ninhydrin (table 2 (i)), and again the growth response indicated that the growth factor was neither T₃ nor T₄. Large excesses of T₄ on paper or in solution were readily destroyed by ninhydrin (table 2, (j)).

As shown in figure 1 further purification of fraction 2 by extraction with various solvents produced 2 additional active fractions (table 2 (k)). The benzene extract was yellow but inactive. The acetone extract was a bright yellow solution and elicited a growth response somewhat weaker than that of the original sample. It was sufficiently pure to be applied in a band 3.5-cm wide on a paper chromatogram, and when developed with solvent 2, no iodine could be detected (fig. 2). The acetone: HCl extract produced a growth response comparable to that of the original sample, but it could not be chromatographed in a sufficiently small area to determine whether iodine was present in this fraction. A weak response was also obtained with the methanol extract. It was observed that the sum of the growth effects produced by the separate fractions was greater than that of the original sample, which suggested that 2 active substances were present in fraction 2.

**DISCUSSION**

That more than one fraction, capable of reinstating growth of growth-arrested rats, was present in duodenal powder was re-

Fig. 2 Paper chromatograms of Fraction 2, thyroxine and of triiodothyronine. Solvent front: the top edge of the paper except as indicated. 0: origin. The growth factor remained at the origin of all chromatograms shown. 1. Thyroxine (2 μg) co-chromatographed with 0.11 ml of Fraction 2 using solvent 1 (0.1 N HCl), 16 hours. 2. Triiodothyronine (2 μg) co-chromatographed with 0.11 ml of Fraction 2 using solvent 2, 16 hours. The solvent front of 1 and 2 is beyond the top edge of these strips. Residual chloride from the solvent also reacted with ceric sulfate-arsenious acid (6) and produced a streaked, mottled appearance on 1 and 2. 3. Thyroxine (2 μg) co-chromatographed with 0.15 ml of Fraction 2 using solvent 2 (methanol:0.2 M ammonium acetate, pH 4.5; 1:1). The slower moving substance is thyroxine. The origin and the 3 sections indicated by the horizontal lines to the right of this chromatogram, were those which were cut out, eluted and assayed as described in the text (also table 2, (f)), 4. This is the image of chromatogram 3. By the technique of Kono et al. (6), the chromatogram, pressed onto another strip of paper saturated with ceric sulfate-arsenious acid, leaves an image of all iodinated compounds on this paper. The band at the origin which is in reality orange, leaves no image, thus demonstrating the absence of detectable amounts of iodine at the origin. This was also true of chromatograms 1 and 2. 5. The acetone extract of Fraction 2 (table 2, (k)) developed with solvent 2. 6. The image of a chromatogram on which 1 μg of triiodothyronine was co-chromatographed with the acetone extract of Fraction 2 using solvent 2. The band at the origin (as seen on 5) was again not detectable indicating that the band at the origin does not contain iodine.
GROWTH FACTOR AND THYROID HORMONES

**TABLE 2**

Growth response of growth-arrested* rats to the growth factors of duodenal powder and to thyroxine after various treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Results*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Growth factor</td>
</tr>
<tr>
<td></td>
<td>with T₄</td>
</tr>
<tr>
<td>g gained/2 weeks</td>
<td>g gained/2 weeks</td>
</tr>
<tr>
<td>None</td>
<td>32</td>
</tr>
<tr>
<td>Benzene extract of fraction 1</td>
<td>40, 43</td>
</tr>
<tr>
<td>Benzene extract plus 5 mg of ninhydrin</td>
<td>38, 39</td>
</tr>
</tbody>
</table>

Assay of fraction 1 (2 ml assayed/rat)

(a) NaOH hydrolysis under nitrogen

(b) Reduced with hydrogen-palladium

(c) Extracted with HCl and benzene

(d) NaOH hydrolysis under nitrogen

(e) Paper chromatography, solvent 1

(f) Paper chromatography, solvent 2

(g) Paper chromatography, solvent 3

(h) Paper chromatography, solvent 3

(i) Heated in solution with ninhydrin

(j) 15 µg of T₄ on filter paper sprayed with ninhydrin

(k) Benzene extract

Acetone extract

Acetone-HCl extract

Methanol extract

Assay of extracts of fraction 2

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*No rat had gained more than 5 g during the 2 weeks prior to its use in an assay.
*Each value represents the 2-week body weight gain of one rat which had been fed the sample treated as indicated. For each rat, the sample was mixed into 15-20 g of the sulfaguanidine-containing diet and this was fed until it was consumed. The sulfaguanidine-containing diet was then fed for the remainder of the 2-week period. The quantity of sample used for each treatment was equal to that of the untreated sample so that the response to the treated sample may be compared to that before treatment.

*The hydrolysis of the insoluble fractions was carried out with 10 µg of T₄, acidification of the soluble and insoluble fractions was carried out with 10 µg of T₄, each section was cut out, extracted and assayed with 2 rats.

Six milliliters of fraction 2, with and without 10 µg of T₄, were developed on paper with methanol:0.2 M ammonium acetate at pH 4.4 (1:1) at 5°. The indicated Rf sections were cut out, extracted and assayed with 2 rats.

Six milliliters of fraction 2, with and without 10 µg of T₄, were developed on paper with methanol:0.2 M ammonium acetate at pH 4.4 (1:1) at 5°. The indicated Rf sections were cut out, extracted and assayed with 2 rats.

Fifteen milliliters of fraction 2 were dried on filter paper and then extracted as indicated. Two-fifths of each extract were assayed with 2 rats.
ported briefly elsewhere. The data presented in this report indicate that 3 active fractions were separated from the acid-insoluble residue of duodenal powder after acid hydrolysis. These were a fraction insoluble at pH 8 and 2 fractions separated from a fraction that was insoluble at pH 6. The possibility exists that these growth factors are chemically modified forms of the same active agent although numerous attempts to isolate a growth factor from duodenal powder has consistently produced from 3 to 5 active fractions depending on the procedure or technique used.

It was concluded that fraction 2 contained neither thyroxine nor triiodothyronine. The stability of the growth factors in this fraction to acid hydrolysis is strong evidence to support this conclusion. Haga (9) reported that 86% of L-thyroxine was decomposed by boiling with HCl for 12 hours, and this decomposition was accelerated if carbonyl compounds such as glucose or xylose were present. The more drastic conditions (5 N HCl for 22 hours) used here and filtration of the acid hydrolysate would be expected to remove most, if not all, thyroid hormones from the acid-insoluble residue. More conclusively, the Rf of the growth factors in fraction 2 on paper chromatograms differed from that of the thyroid hormones. In addition, ninhydrin did not destroy the growth factors as it did thyroxine.

The metabolic significance and the relationship of the growth factors in duodenal tissue to thyroid hormone function remains to be determined.

**LITERATURE CITED**


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