larger amount of albumen at the disposal of the embryo is of special value in giving it a better start in life outside the capsule.

Three years of laboratory culture of *Ancylus fluviatilis* Müller have shown that this species has a higher percentage of abnormalities (20%) in the eggs than any other species examined. The frequency of the different kinds of abnormal eggs and egg cells is shown in Table 1.

Most common in all three years are dwarf eggs, smaller than ordinary size (1.42 × 1.20 mm). Dwarf eggs may be a phenomenon definitely connected with the starting or stopping of oviposition. In both series of eggs, those throughout the egg-laying period and those in a single capsule, dwarf eggs are found mainly at the end of each series, but partly at the beginning. The percentages in which they occur in different positions within the capsules are given in Table 2.

The considerable number of dwarf eggs and other irregularities given in Table 1 also make *Ancylus fluviatilis* Müller an interesting subject for studies of the earliest phases of embryonic development. Even when still *in ovo* the embryos may meet with various influences that interfere with their normal development—e.g., variations in the quantity of albumen, crowding because of abnormal development of a neighboring embryo or accidental formation of "nurse eggs" (competition), or abnormal development of the egg cell itself, determined by intrinsic factors (e.g., lack of fertilization) or extrinsic factors (e.g., crushing of the egg cell during the formation of the capsule).

The advantages of the egg capsules of *Ancylus fluviatilis* Müller as experimental material can be summarized as follows. The limpet is a suitable animal for studying oviposition and embryogenesis under laboratory conditions. The apparatus described makes it possible to obtain living eggs, laid in transparent capsules, for microscopic investigation of living preparations. The capsule is easily turned, and the eggs can be studied in series exactly in the order in which they were laid. The frequency of abnormalities of several sorts in the eggs make the capsules particularly well suited for studies of cell physiology and other aspects of experimental biology.

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Presence of Iodinated Amino Acids in Unhydrolyzed Thyroid and Plasma[1]

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De Robertis (*1*) demonstrated the presence of a proteolytic enzyme in the colloid of the thyroid follicle. He postulated that this enzyme causes the breakdown of thyroglobulin with the release of biologically active fragments which are of low enough molecular weight to diffuse out of the follicle, and which presumably constitute the thyroid hormone. The only compound of small molecular weight found so far in unhydrolyzed thyroid is thyroxine (*4*). In the present work, the technique of paper chromatography has been used to confirm this result, and to study the production of other iodinated amino acids which might be formed during the breakdown of thyroglobulin. The appearance of these substances in the circulation has been studied by observing their presence in *n*-butanol extracts of plasma. In addition, chromatographic analysis of whole plasma has supplied evidence as to the nature of the circulating thyroid hormone.

Methods. Female albino rats weighing 150–200 g and maintained on Remington's low iodine diet No. 342 (*5*), to which 10% brewers' yeast had been added, were injected subcutaneously with about 100 μc of carrier-free radioactive iodide (NaI^{131}). After 48 hours the animals were anesthetized with ether and exsanguinated with a heparinized syringe via the inferior vena cava.

Chromatography of n-butanol extracts of thyroid and plasma. The thyroids were removed immediately, ground in 1 ml of ice-cold saline in a chilled mortar, and extracted three times with an equal volume of *n*-butanol. Similarly, the plasma was extracted three times, first with a double volume and then twice with an equal volume of butanol. In both cases, the combined butanol extracts were reduced to dryness *in vacuo* at room temperature. The dry residue was taken up in distilled water, 0.1 ml and 0.2 ml being used for the thyroid and plasma residues, respectively.

Aliquots of 0.02 ml of this solution were then chromatographed by the capillary ascent method of Williams and Kirby (*10*), with or without the addition of 20 μg of each of the following carriers: DL-thyroxine, DL-diiodothyronine, DL-diiodotyrosine, and DL-monoiodotyrosine. The aliquots were placed in the lower left-hand corner of 10 in. × 12 in. Whatman No. 1 filter paper sheets and

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dried. The developing solvent used for the first dimension was the upper layer obtained after shaking *n*-butanol with 2*N* formic acid in a separatory funnel. For the second dimension, the upper layer of a mixture of 4 parts *n*-butanol and 1 part dioxane shaken with 5 parts of 2*N* NH_4OH was used. After overnight development for each dimension, the papers were removed and dried in a current of air. They were then applied to Eastman No-Screen x-ray film, with a sheet of cellophane intervening, and thus exposed for varying periods of time. The duration of exposure depended on the activity present, as determined with a Geiger probe monitor. After autography (Figs. 1–3) the papers were dried for 30 min at 100° C, sprayed with ninhydrin (0.1% solution in $CHCl_3$ with the addition of 0.1% collidine), and dried at 100° C again. The colors obtained with ninhydrin were rather pale, but were sufficiently clear to locate the added carrier amino acids. By this technique it was possible to obtain a complete separation of the four iodinated amino acids and of iodide as well.

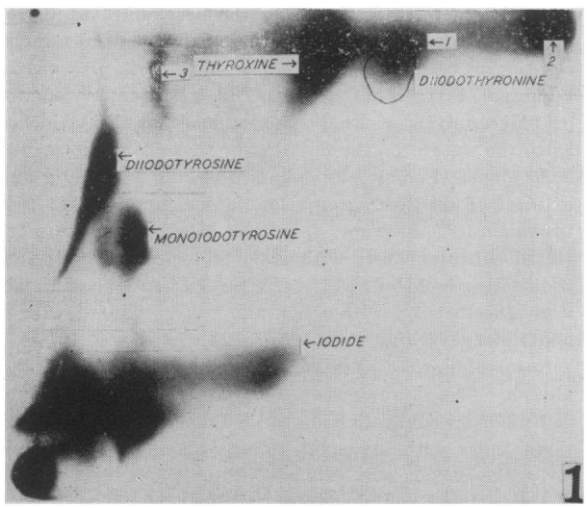

FIG. 1. An autograph of a two-dimensional chromatogram of the entire butanol extract of the fresh thyroid taken from an adult rat given 100 μc of carrier-free NaI^{131} subcutaneously, 48 hr previously. Twenty μg each of nonradioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine, and DL-diiodothyronine were added to the butanol extract. The paper was developed vertically with a butanol-formic acid mixture and horizontally with a butanol-dioxane-ammonia mixture. The material was deposited on the left lower corner. Some spots are due to thyroxine, diiodotyrosine, monoiodotyrosine, and iodide; others are due to unidentified substances indicated by the numbers 1, 2, and 3.

Chromatography of the plasma proteins. The plasma proteins, combined with hemin in the manner described by Franklin and Quastel (*2*), were chromatographed at pH 7 using M/20 citrate buffer in the first dimension and M/20 phthalate buffer in the second dimension. Two hours were allowed for a satisfactory development in each dimension. After the paper had dried, the benzidine reagent was applied with a small paint brush, resulting in the formation of a blue color at the site of the plasma protein. The results were photographed immediately.

This method was used with radioactive plasma obtained from the radioiodide-treated rats described. Plasma

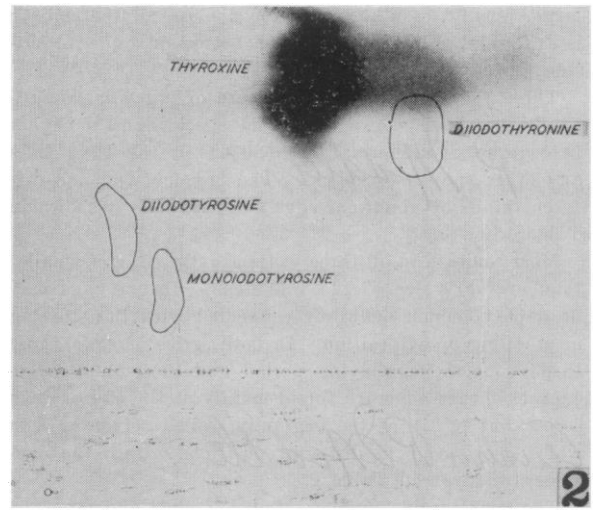

FIG. 2. An autograph of the entire butanol extract of 2.5 ml of the plasma of the same animal as in Fig. 1; the same carriers were added. An intense thyroxine spot is visible. The much less intense spot next to it is probably due to the substances causing spot 1 in Fig. 1.

(0.02 ml) was applied directly to the paper. After the two-dimensional chromatography, the paper was placed in contact with an x-ray film for autography (Fig. 5) and then treated with benzidine for localization of the proteins (Fig. 4).

The remaining plasma was extracted with butanol, and the butanol was divided into two portions which were dried in the usual manner. The first portion was dissolved in 0.1 ml of 4% NaCl, of which 0.01 ml was chro-

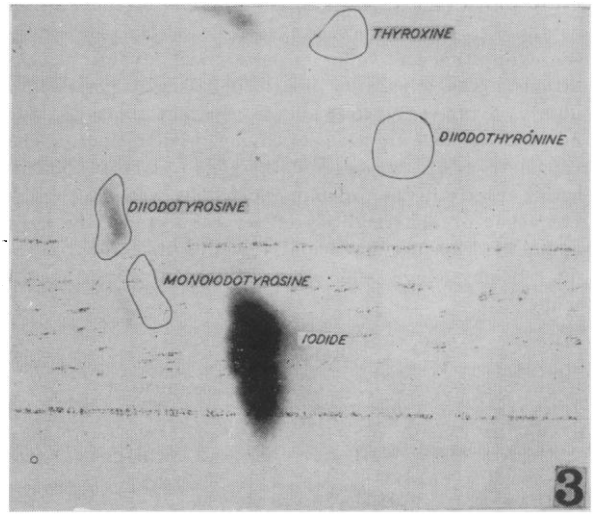

FIG. 3. An autograph of a control chromatogram of a mixture containing carrier-free NaI^{131}, and 20 μg each of nonradioactive DL-monoiodotyrosine, DL-diiodotyrosine, DL-thyroxine, and DL-diiodothyronine. Besides an intense spot due to iodide, a faint spot is visible in the location of diiodotyrosine. Two spots due to unidentified substances are also present. When the marked intensity of the iodide spot is compared with the low intensity of all other spots, it is apparent that only a very small percentage of the iodide present exchanged with diiodotyrosine and none at all with thyroxine.

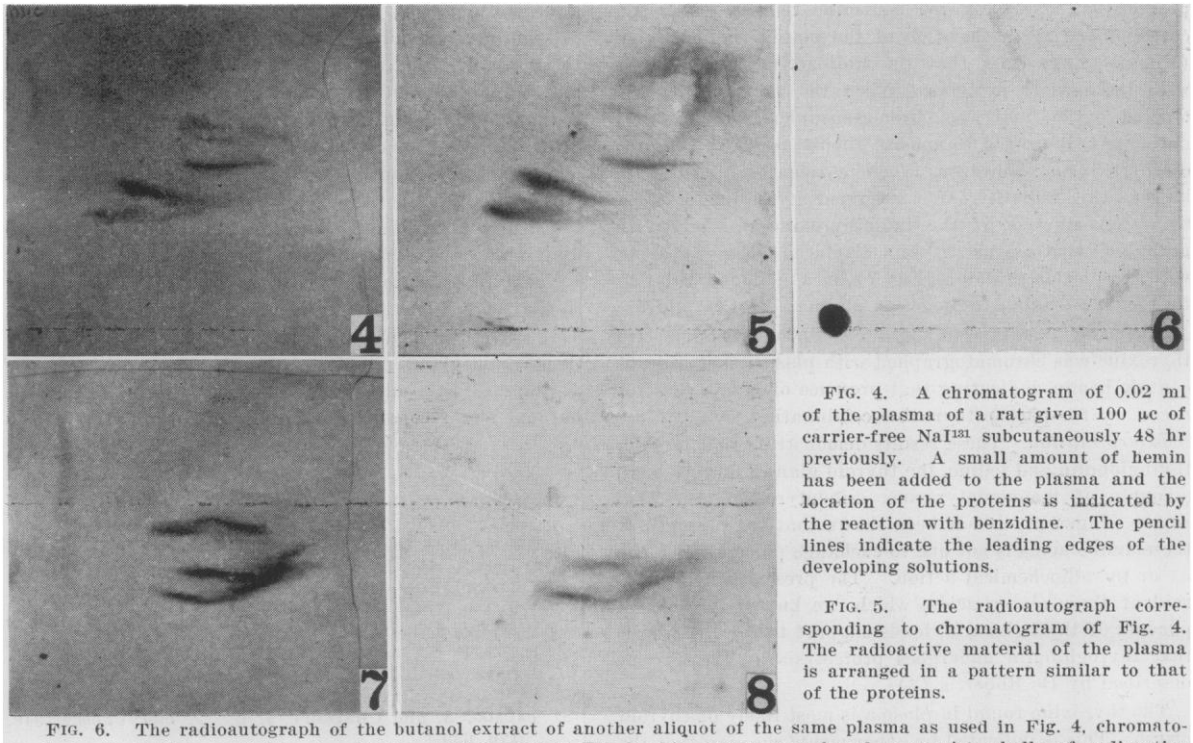

FIG. 4. A chromatogram of 0.02 ml of the plasma of a rat given 100 μc of carrier-free NaI^{131} subcutaneously 48 hr previously. A small amount of hemin has been added to the plasma and the location of the proteins is indicated by the reaction with benzidine. The pencil lines indicate the leading edges of the developing solutions.

FIG. 5. The radioautograph corresponding to chromatogram of Fig. 4. The radioactive material of the plasma is arranged in a pattern similar to that of the proteins.

FIG. 6. The radioautograph of the butanol extract of another aliquot of the same plasma as used in Fig. 4, chromatographed with buffers as in Fig. 4. By the method used in Fig 2, this extract was found to consist wholly of radioactive thyroxine. The radioactive thyroxine did not move from the origin.

FIG. 7. A chromatogram of 0.02 ml of nonradioactive plasma, to which hemin had been added and combined with an aliquot of the butanol extract used in Fig. 6, chromatographed with buffers as in Fig. 4, and sprayed with benzidine. The protein pattern is apparent.

FIG. 8. The radioautograph corresponding to chromatogram of Fig. 7. The radioactive thyroxine added to nonradioactive plasma has moved with the proteins, giving a picture similar to that obtained with radioactive plasma (see Figs. 4 and 5).

matographed with the butanol developers and autographed. Another 0.01-ml aliquot was chromatographed with buffers and autographed (Fig. 6). Finally, the second portion of dried butanol extract was dissolved in nonradioactive plasma obtained from another rat, mixed with hemin, chromatographed with buffers (Fig. 7), and autographed (Fig. 8).

Confirmatory experiments with plasma proteins not containing hemin were carried by the addition of nonradioactive DL-thyroxine. Thyroxine (100 μg) was mixed with 0.02 ml radioactive plasma, chromatographed with buffers and the location of thyroxine determined by spraying with ninhydrin.

Identification of components. Thyroid and plasma fractions of ten animals were chromatographed and autographed. The autographs (Fig. 1) of the butanol extract of thyroid, with added carriers, showed the presence of spots corresponding to monoiodotyrosine, diiodotyrosine, thyroxine, and iodide; all of which were found in the ten animals investigated. These spots were similar in location and intensity when no carrier was used, and therefore the presence of these substances cannot be due to an exchange between the labeled iodide and the carriers. This was borne out by control experiments in which tracer iodide was chromatographed with carriers (Fig. 3). In addition, several compounds, whose identities are unknown, were found more or less regularly throughout the series of thyroid chromatograms. Thus, of the unknowns marked 1, 2, and 3 (Fig. 1), compound 1 was found in ten and the other two compounds in seven of the preparations.

Thyroxine made up the greater part of the radioactivity of the butanol extract of plasma (Fig. 2). By using the entire plasma extract and exposing for a long period of time, it was possible to distinguish faint spots corresponding to iodide in five plasma samples and to diiodotyrosine in six. Both substances were found together in extracts of five samples of plasma. The results were identical whether or not carriers were added to the plasma extract.

A possible source of error lay in the production of artifacts by the radiochemical effect of the large doses of radioactivity used. However, it could be demonstrated that the same amino acids were present in thyroid and plasma of two rats in which radioactivity of the injected radioiodide was only 1 μc, a dose which has been shown to have no effect on the physiological processes of the thyroid (7). A very long exposure had to be used to detect the minute amounts of radioactivity present in the chromatographic spots obtained under these conditions.

Nature of the circulating thyroid hormone. When an aliquot of whole plasma from an animal given radioactive iodide was mixed with hemin and chromatographed using buffer solutions for development, the distribution of the

proteins, as shown by the benzidine reagent (Fig. 4), corresponded to the location of the plasma radioactivity (Fig. 5), indicating that the radioactivity had moved with the plasma proteins. When the radioactivity extracted by butanol was chromatographed with butanol developers, it proved to consist almost exclusively of radiothyroxine. However, when chromatographed with buffers, this radiothyroxine remained at the origin (Fig. 6). Nevertheless, if the radiothyroxine was added to nonradioactive plasma, it was displaced (Fig. 8) along with the plasma proteins (Fig. 7), in a manner similar to that of the original radioactive plasma (Figs. 4 and 5).

The same results were obtained when nonradioactive thyroxine was chromatographed with plasma that did not contain hemin, indicating that presence of hemin was not necessary for plasma-thyroxine combination.

Interpretation. These results demonstrate that, besides thyroglobulin and iodide, the thyroid gland contains small amounts of monoiodotyrosine, diiodotyrosine, and thyroxine (Fig. 1). It has been shown that the presence of these amino acids is not due to exchange phenomena (Fig. 3) or to radiochemical action. The presence in the thyroid of these amino acids, which are known to be components of thyroglobulin, lends support to the hypothesis that thyroglobulin undergoes proteolysis by the enzyme described by De Robertis (*1*).

The thyroxine found in plasma is most likely of thyroid origin. This is indicated by experiments showing that the butanol-soluble radioactivity of plasma is extremely low in thyroidectomized rats (*3*). The passage of thyroxine into the circulation has been previously explained by a gradient of thyroxine concentration in thyroid tissue and plasma (*4*). It may be pointed out further that the diffusion of thyroxine from the thyroid would tend to correct the increase in osmotic tension due to proteolysis in the colloid. The failure to find significant amounts of the other iodinated amino acids in plasma may be explained by their destruction within the follicle and a reutilization of their iodine by the gland.

In the plasma, thyroxine has been found combined with proteins. This combination may be severed easily by butanol, but is reconstituted when thyroxine is placed in contact with plasma proteins (Figs. 7 and 8). Furthermore, since plasma proteins may combine with amounts of thyroxine well above the physiological doses (100 μg in 0.01 ml), it may be assumed that under physiological conditions the thyroxine secreted from the thyroid combines with plasma proteins as it is released.

The nature of the thyroxine complex in plasma is obscure. However, iodine has been found in the albumin fraction of plasma (*6, 8, 9*), and to a somewhat lesser extent in the globulin fraction (*6, 8*). Attempts to determine the plasma constituent which is the thyroxine carrier are currently under way.

In summary, when adult female rats on a low iodine intake were sacrificed 48 hr after an injection of carrier-free radioiodide, butanol extracts of unhydrolyzed thyroid and plasma analyzed by radioautography of two-dimensional paper chromatograms revealed the presence of six radioactive compounds besides iodide and thyroglobulin. Of these, three were identified as thyroxine, diiodotyrosine, and monoiodotyrosine. In the blood plasma, practically all the butanol-extractable radioactivity was present as thyroxine, with a very low amount of diiodotyrosine and iodide. When the whole plasma was analyzed by radioautography of two-dimensional buffer chromatograms, it was shown that the location of the radioactive material corresponded to that of the plasma proteins. Although thyroxine solutions showed no movement from the origin under these conditions, radioactive thyroxine dissolved in nonradioactive plasma was displaced along with the plasma proteins and gave a pattern very similar to that of radioactive plasma itself.

It was concluded that thyroxine, after its release by the thyroid gland, circulates in combination with plasma proteins. The complex thus formed can be split with butanol and reconstituted *in vitro*.

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Xanthopterin Obtained from the Skins of the Yellow Mutant of *Bombyx mori* (Silkworm)

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The gene named "Lemon colored" which is present in the third chromosome of *Bombyx mori* gives rise to yellow-colored larvae instead of the white ones of the normal type. The yellow pigment exists in the epidermis and is considered to possess a close relationship with tryptophan metabolism, as in the case of the eye pigments of *Drosophila* and other insects (*4, 5*). Chemical researches made on this pigment have identified it as xanthopterin.

A hundred larvae of the yellow *lem* type in the fifth instar were dissected, the skins were denatured by treatment with alcohol, the lipochromes were extracted with ether in a Soxhlet apparatus, and the pigments finally were extracted with 200 ml and 400 ml of boiling water. The first extract gave a purple-plus-yellowish green fluorescence, and the second a purple fluorescence. The first extract was made acidic (0.05*N*) with hydrochloric acid and left overnight in an icebox, when 160 mg of white crystals was obtained. These crystals, after recrystalliza-

[1] The chemical researches of this report were carried out by Hirata and Nakanishi.

[2] The authors wish to acknowledge the helpful suggestions of Prof. F. Egami during the course of this research.