

## The Formation, Distribution and Function of Ribosomes and Microsomal Membranes during Induced Amphibian Metamorphosis

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1. A lag period of about 4 days preceded the onset of metamorphosis precociously induced by tri-iodothyronine in tadpoles of the giant American bullfrog (*Rana catesbeiana*). It was established by the accelerated synthesis or induction of carbamoyl phosphate synthetase and cytochrome oxidase in the liver, serum albumin and adult haemoglobin in the blood, acid phosphatase in the tail, and the increase in the hindleg/tail length ratio. 2. A 4- to 6-fold stimulation, 2 days after the induction of metamorphosis, of the rate of synthesis of rapidly labelled nuclear RNA in liver cells was followed by an increasing amount of RNA appearing in the cytoplasm. Most of the newly formed RNA on induction of metamorphosis was of the ribosomal type. An accelerated turnover at early stages of development preceded a net accumulation of RNA in the cytoplasm, with no change in the amount of DNA per liver. 3. Most hepatic ribosomes of the pre-metamorphic tadpoles were present as 78s monomers and 100s dimers; metamorphosis caused a shift towards larger polysomal aggregates with newly formed ribosomes that were relatively more tightly bound to membranes of the endoplasmic reticulum. 4. The appearance of new polyribosomes in the cytoplasm on induction of metamorphosis was co-ordinated in time with a stimulation of synthesis of phospholipids of the smooth and rough endoplasmic reticulum, followed by a gradual shift in preponderance from the smooth to the rough type of microsomal membranes. 5. Electron- and optical-microscopic examination of intact hepatocytes revealed a striking change in the distribution and nature of ribosomes and microsomal membranes during metamorphosis. 6. Ribosomes prepared from non-metamorphosing and metamorphosing animals were identical in their sedimentation coefficients and in the structural ribosomal proteins. The base composition and sedimentation coefficients of ribosomal RNA were also identical. Induction of metamorphosis also did not alter the incorporation of  $^{32}\text{P}$  into the different phospholipid constituents of microsomal membranes. 7. Nascent  $^{14}\text{C}$ -labelled protein with the highest specific activity was recovered in the 'heavy' rough membrane fraction of microsomes, whereas little  $^{14}\text{C}$  was associated with 'free' polysomes. Protein synthesis *in vivo* was most markedly stimulated during metamorphosis in the tightly membrane-bound ribosomal fraction after the appearance of new ribosomes. 8. The rate of synthesis of macromolecules *in vivo* could not be followed beyond 7-8 days after induction because of variable shifts in precursor pools due to regression of larval tissues. 9. The stimulation of RNA and ribosome formation was specifically associated with the process of metamorphosis since no similar response to thyroid hormones occurred in those species (*Axolotl* and *Necturus*) in which the hormones failed to induce metamorphosis.

The fact that metamorphosis can be induced precociously in many amphibia by exogenous thyroid hormones has made it an attractive system for studying the regulation of late embryonic developmental changes (for reviews see Brown & Cohen, 1958; Bennett & Frieden, 1962; Weber,

1963; Cohen, 1966). It is well established that in anuran tadpoles both the hormone-induced and spontaneous processes involve the initiation of synthesis of specific proteins accompanied by dramatic structural alterations in almost every type of cell. The induction of protein synthesis by

these hormones is unlike many adaptational processes, since it occurs without any change in the environment of the larva and includes such diverse substances as urea-cycle enzymes, serum albumin, deoxyribonuclease, collagenase, rhodopsin and adult haemoglobin.

In addition to the obvious advantages of inducing developmental processes in independently surviving embryos, I was led to study metamorphosis in amphibia to complement my work on the action of growth and developmental hormones in mammals. Although my earlier work on the action of thyroid hormones in young mammals suggested a selective regulation of the synthesis of proteins and cell structures, it was not possible to prove that the hormone actually induced their synthesis (Tata *et al.* 1963; Tata, 1964, 1966a; Gustafsson, Tata, Lindberg & Ernster, 1965; Roodyn, Freeman & Tata, 1965). It is for this reason that I decided to study the precocious induction of anuran metamorphosis by exogenous thyroid hormones. Tadpoles of the giant American bullfrog, *Rana catesbeiana*, were chosen for this purpose for three reasons: (a) the large size of the larvae, which yield 250–450 mg. of liver from each tadpole; (b) the fact that all metamorphic changes could be induced in this species 12–18 months before spontaneous metamorphosis made the possibility of hormonal release of pre-formed constituents less likely; (c) much of the work on the hormone-induced synthesis of proteins of vital importance in embryonic development, such as carbamoyl phosphate synthetase (Paik & Cohen, 1960; Tatibana & Cohen, 1964), serum albumin (Herner & Frieden, 1960) and adult haemoglobin (Bennett & Frieden, 1962; Moss & Ingram, 1965), has been performed in this species of frog.

Initially the design of my experiments on metamorphosis paralleled those on the early effects of thyroid and other hormones on RNA synthesis in the rat (Tata & Widnell, 1966; Widnell & Tata, 1966). Since, at the time I began these studies, there was only one report on overall RNA synthesis in bullfrog tadpoles (Finamore & Frieden, 1961), much of our early work involved the measurement of rates of synthesis of RNA in the nucleus and its transfer to the cytoplasm in non-metamorphosing larvae. During this work, my ideas of the special importance attached to the control of protein synthesis by m-RNA\* in nucleated cells have been modified. In particular it is now believed that RNA transport into the cytoplasm, modulation of translation by ribosomes and changes in the structural environment of the ribosomes are equally important (Henshaw, Bojarski & Hiatt, 1963; Girard, Latham, Penman & Darnell, 1965;

Henshaw, Revel & Hiatt, 1965; Dallner, Siekevitz & Palade, 1966; Tata, 1967a). This shift in emphasis, from the synthesis of m-RNA to its transport and utilization in the cytoplasm, in understanding regulatory mechanisms led me to focus attention in my later studies on the properties and turnover of ribosomes and the membranes of the endoplasmic reticulum.

The aim of the present paper is to show that the induction of metamorphosis in the bullfrog results in complex changes in the turnover of ribosomes, changes in their attachment to membranes and in microsomal protein and phospholipid synthesis. Preliminary results of the turnover of nuclear and cytoplasmic RNA at the onset of metamorphosis have already been described (Tata, 1965, 1967a).

## EXPERIMENTAL

*Animals.* Bullfrog (*Rana catesbeiana*) tadpoles, 7–10 cm. in length with no hind- or fore-limbs, were obtained at first from Lemberger and Co., Oshkosh, Wis., U.S.A., and later from Connecticut Valley Biological Supply Co., Southampton, Mass., U.S.A. Stock animals were maintained at 12–15° in aerated tap water on a diet of chopped spinach and nettle powder. Spontaneous metamorphosis would not occur under these conditions for 12–18 months. Experimental animals were maintained at 18–20° in batches of 8–12 in 3 l. of water. Temperature has been shown to be an important factor governing the rates of morphological and biochemical changes during thyroid-hormone-induced metamorphosis (Paik & Cohen, 1960; Frieden, Wahlborg & Howard, 1965). Mud-puppies (*Necturus maculosus*) were also obtained from the same suppliers as the bullfrog tadpoles, and axolotls (*Ambystoma mexicanum*, albino strain) were kindly supplied by Professor D. Newth; these neotenic amphibians were maintained at 12–15° and fed on a diet of ox heart and live *Tubifex* worms, twice a week.

*Treatment.* Metamorphosis was induced by the injection (intraperitoneal, via the dorsal junction between the tail and the abdomen) of 0.5–1 µg. of 3,3',5-tri-iodo-L-thyronine, after which the animals were placed in water containing 5–10 µg. of tri-iodothyronine/l. This procedure was adopted to eliminate differences in the rates of absorption of the hormone by different routes (see Frieden & Westmark, 1961). Although 3,3',5-tri-iodo-L-thyronine was always used to induce metamorphosis, we checked in our preliminary experiments that the results were qualitatively similar to those obtained with a two- to three-fold greater dose of L-thyroxine. The hindlimb/tail length ratio was used as the morphological index of metamorphosis in long-term experiments. *Necturus* and axolotl larvae were given single or repeated intraperitoneal injections. All isotopically labelled substances were also injected by the intraperitoneal route.

*Subcellular fractionation.* Hepatic nuclei were prepared as described by Widnell & Tata (1964) in a modified homogenization medium containing 0.33 M-sucrose, 10 mM-tris-HCl buffer, pH 7.5 at 18°, 50 mM-KCl and 5 mM-MgCl<sub>2</sub>. Tadpole livers were pooled from 8–16 animals (average liver wt. 200–300 mg.) and homogenized with only six to eight strokes in a 30 ml. loose-fitting (clearance 0.25 mm.)

\* Abbreviations: m-RNA, messenger RNA; r-RNA, ribosomal RNA.

Teflon-glass homogenizer at 400 rev./min. (It is very important not to homogenize the tissue at higher speeds or for a longer duration, to minimize degradation of nuclei and release of DNA into the cytoplasmic fractions.) Polyvinyl sulphate (0.1%) was included to minimize breakdown of RNA and polysomes. The yield of nuclei was 40–50%, on the basis of DNA recovery. Mitochondria, microsomes and the post-microsomal supernatant were separated from the initial nuclei-free homogenate by differential centrifugation (Tata *et al.* 1963) and contained less than 2–4% of total DNA. Pellets of the particulate fractions were usually suspended in a small volume of the homogenizing medium. Ribosomes were prepared by treating post-mitochondrial supernatants with 0.1–0.4% sodium deoxycholate before centrifuging at 105 000g for 50–100 min. The following procedure was adopted for fractionating microsomes without the use of detergents: livers were homogenized in 2.5–3.0 vol. of a medium containing 0.35 M-sucrose, 50 mM-tris-HCl buffer, pH 7.6 at 18°, 25 mM-KCl and 10 mM-MgCl<sub>2</sub> and centrifuged at 7500g for 12 min. Then 5.5 ml. of the post-mitochondrial supernatant, after a second centrifugation at 7500g, was layered over 2.5 ml. of 1.3 M-sucrose containing the same buffer and salts in a 10 ml. tube of an MSE 3 × 10 ml. swing-out rotor and centrifuged at 105 000g for 120 min. in an MSE Superspeed 50 ultracentrifuge. The suspension of smooth microsomal membranes at the interface (Dallner, 1963) was drawn off and the pellet of rough endoplasmic reticulum suspended in 1 ml. of the 0.35 M-sucrose medium. The suspension was layered over a discontinuous gradient of 3.0 ml. of 2.0 M-sucrose and 3.5 ml. of 1.5 M-sucrose with buffer and salts as above and centrifuged at 105 000g for 3.5 hr. in a 3 × 10 ml. swing-out rotor of the MSE Superspeed 50 ultracentrifuge. The layers of rough membranes at the interfaces of 1.5 M- and 2 M-sucrose were removed and designated as 'light' and 'heavy' rough membranes respectively. The pellet, containing largely glycogen with free polysomes and ribosomes (Bloemendal, Bont & Meisner, 1966), was rinsed and suspended in 0.35 M-sucrose medium. All subcellular fractionation procedures were carried out at 2°.

**Polyribosomal profiles.** Ribosomes obtained by sodium deoxycholate treatment of a mitochondria-free supernatant from about 500 mg. of liver were layered over a linear 15–30% (w/v) sucrose gradient containing 50 mM-KCl, 20 mM-tris-HCl buffer, pH 7.6, and 1.5 mM-MgCl<sub>2</sub>. The gradient was prepared over 3 ml. of 60% (w/v) sucrose with buffer and salts and centrifuged at 22 000 rev./min. for 2.5 hr. in a Spinco SW25.1 rotor. Fractions (1 ml.) were collected and processed for labelled RNA and protein as described by Tata & Widnell (1966), after adding 250 µg. of carrier yeast RNA and serum albumin to each tube.

**Labelling of nuclear and cytoplasmic RNA and protein.** Bullfrog tadpoles were injected with 5–10 µC of [<sup>3</sup>H]-uridine, 1–2 µC of [<sup>14</sup>C]orotic acid or 5–10 µC of [<sup>32</sup>P]-phosphate and the animals were killed at different time-intervals to determine the rate of labelling of RNA. Higher doses of the radioactive compounds were used for *Necturus* and axolotls. The specific activity of radioactive RNA present in the different subcellular fractions was determined as described by Tata & Widnell (1966). <sup>14</sup>C and <sup>3</sup>H were measured in a Packard Tri-Carb scintillation spectrometer, with efficiencies of 66% and 40% respectively, in a fluid comprising 0.4% 2,5-diphenylloxazole, 0.01% 1,4-bis-(4-methyl-5-phenylloxazol-2-yl)benzene and 18% naphthalene

(nuclear grade) in redistilled dioxan. <sup>32</sup>P was measured in a Nuclear-Chicago thin-window gas-flow counter (efficiency about 40%).

To study the rate of incorporation of amino acids into protein *in vivo*, tadpoles at different stages of metamorphosis were injected with 1–3 µC of a mixture of <sup>14</sup>C-labelled amino acids (*Chlorella* protein hydrolysate) between 10 and 45 min. before they were killed. The particulate fractions were suspended in water except for the microsomes and their subfractions, which were suspended in the homogenizing medium containing 0.4% sodium deoxycholate, and the proteins precipitated with 5% (w/v) trichloroacetic acid. The precipitate was filtered on Oxoid (Oxo Ltd., London, E.C. 4) membrane filters and washed twice with 5% trichloroacetic acid at 5°, 5% trichloroacetic acid at 80° and water, in that order. The dried filters were used for radioactivity measurement and the protein contents of samples of the particulate suspension and of the cell sap were determined separately.

**Labelling of membrane phospholipids.** Phospholipids were labelled with 5–10 µC of [<sup>32</sup>P]phosphate or 1–2 µC of [*Me*-<sup>14</sup>C]choline chloride per tadpole. They were precipitated from the particulate fractions with 0.4 N-HClO<sub>4</sub> or 5% trichloroacetic acid and washed three times with the acid before extraction with chloroform-methanol (2:1, v/v) (two extractions at 20° and two at 45°). The organic solvent extracts were washed twice with 0.1 N-HCl and water before being evaporated to dryness under vacuum at 40°. The identity of phospholipids was checked by thin-layer chromatography on silica gel in chloroform-methanol-acetic acid-water (De Graeff, Dempsey, Lameyer & Leaf, 1965). The distribution of radioactivity in the different phospholipids was measured by eluting the respective chromatogram spots or by a double-beam micro-densitometer (Joyce-Loebl) scan of a radioautograph of the chromatogram. The amount of total phospholipid was calculated as 25 times the phosphorus content (Ansell & Hawthorne, 1964).

**Enzyme assays.** Carbamoyl phosphate synthetase (ATP-carbamate phosphotransferase, EC 2.7.2.2) in tadpole livers was assayed as described by Brown, Brown & Cohen (1959). Cytochrome oxidase (cytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1) in isolated mitochondria was assayed as described by Tata *et al.* (1963). Acid and alkaline phosphatases (orthophosphoric monoester phosphohydrolases, EC 3.1.3.1 and EC 3.1.3.2) were assayed in homogenates and supernatant extracts of both liver and tail as described by Frieden & Mathews (1958), but with *o*-nitrophenol phosphate as the substrate. Specific activities of these enzymes were expressed per mg. of tail protein in the extract.

**Blood proteins.** The induction by tri-iodothyronine of the synthesis of serum albumin and adult haemoglobin was studied by paper electrophoresis in veronal buffer, pH 8.6 (Herner & Frieden, 1960, 1961), or starch-gel electrophoresis as described by Baglioni & Sparks (1963).

**Sedimentation constants of ribosomes and polyribosomes.** The ribosomal pellet obtained by deoxycholate treatment of the mitochondria-free supernatant was homogenized in 2 ml. (0.4 mg./ml.) of 50 mM-tris, 50 mM-KCl and 10 mM-MgCl<sub>2</sub> and centrifuged at 0° in the Spinco model E ultracentrifuge. Glycogen, which is of largely high molecular weight (> 100s), was not removed from the ribosomal suspension.

*Extraction and electrophoresis of ribosomal proteins.* This procedure was carried out by Dr Mary G. Hamilton at the Sloan-Kettering Institute, New York. Ribosomal pellets were flown to New York in solid CO<sub>2</sub> and suspended in 50 mM-tris-HCl buffer, pH 7.4, and 50 mM-KCl without Mg<sup>2+</sup> to dissociate the ribosomal sub-units as well as release loosely bound proteins and transfer RNA. Much of the glycogen was removed by centrifuging at 105 000g for 5–10 min. before sedimenting the ribosomal sub-units. The pellets were re-suspended in tris-KCl medium, LiCl was added to a concentration of 2 M and the mixture was kept in the cold for more than 3 days. The supernatant containing ribosomal proteins was dialysed against 6 M-sodium acetate, pH 5.6, to remove LiCl and then concentrated to a small volume against Sephadex G-200. The ribosomal proteins were resolved by disk electrophoresis in polyacrylamide gel in 6 M-urea, pH 4.3, according to the method of Reisfeld, Lewis & Williams (1962). The proteins were stained and their relative distribution was determined with an automatic recording densitometer.

*Base composition of RNA.* RNA was hydrolysed in 0.4 N-KOH and the ribonucleotides were separated by ion-exchange chromatography as described by Katz & Comb (1963). In some experiments the nucleotides were separated by the paper-chromatographic method of Lane (1963) and the relative amount of radioactivity was determined from a densitometric scan of radioautograms (Ilford No-Screen X-ray film).

*Extraction of RNA.* Both nuclear and ribosomal RNA were extracted with phenol and 1% sodium dodecyl sulphate as described by Hiatt (1962), with the exception that nuclear RNA was extracted at 55° for 5 min. The preparation was suspended in 50 mM-sodium acetate buffer, pH 5.0, and 50 mM-NaCl with 25–50 mg. of bentonite and macaloid/ml. before extraction to minimize degradation of the RNA.

*Chemical determinations.* Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as the standard. DNA was estimated by the method of Burton (1956) and RNA by the method of Ceriotti (1955) with calf thymus DNA (Sigma) and yeast RNA (Boehringer) as standard. Phosphorus was measured by the method of Fiske & Subbarow (1925), or by the adaptation by Ames & Dubin (1960) of the method of Chen, Toribara & Warner (1956). Phospholipid was estimated as 25 × phospholipid phosphorus.

*Materials.* All chemicals were of analytical grade and organic solvents were distilled before use. Enzymes, nucleotides, phospholipids and other reagents were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., or C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. L-Thyroxine and 3,3',5-tri-iodo-L-thyronine were obtained from Glaxo Laboratories, Greenford, Middlesex, and dissolved in aq. 1% (w/v) propane-1,2-diol, pH 8.4. Carrier-free [<sup>32</sup>P]phosphate, [6-<sup>14</sup>C]orotic acid (6.8 mc/m-mole), [<sup>3</sup>H]uridine (1250 mc/m-mole), [Me-<sup>14</sup>C]choline chloride (32 mc/m-mole) and U-<sup>14</sup>C-labelled protein hydrolysate (640 mc/mg.) were obtained from The Radiochemical Centre, Amersham, Bucks. The last-named product was freed from any contaminating radioactive nucleotides by ion-exchange chromatography.

*Microscopical methods.* For electron microscopy small

pieces of tadpole liver were fixed by immersion for 1 hr., with either a chilled 1% osmium tetroxide fixative with balanced salts added (Zetterqvist, 1956) or a solution of 5% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, before treatment with osmic acid. The specimens were dehydrated with ethanol and embedded in Epikote epoxy resin. Thin sections were collected on uncoated copper grids and stained with uranyl acetate and with lead citrate (Reynolds, 1963). For light microscopy larger portions of the liver were fixed in a saturated solution of HgCl<sub>2</sub> with 4% acetic acid, and embedded in paraffin wax. Sections were stained with toluidine blue or with haematoxylin and eosin.

## RESULTS

### *Time-course of acceleration of total or specific protein synthesis on induction of metamorphosis*

To choose the most meaningful period for studying regulatory mechanisms, it was initially necessary to establish the time-course of change in the content of total or specific proteins after the precocious induction of metamorphosis. Fig. 1 shows the changes in the total protein content of the major subcellular fractions of tadpole liver during the first 10 days after induction of metamorphosis with tri-iodothyronine. After a lag period of 3–4 days there was an increase in total protein content expressed per g. wet wt. of the liver. Much of the initial increase in total tissue protein concentration could be ascribed to dehydration of the tissue, as it was less marked when expressed on the basis of DNA content (there was no increase in DNA content of the organ for 8–9 days after metamorphosis was induced). Dehydration of many tissues is a characteristic feature of amphibian metamorphosis (see Etkin, 1964; Lewis & Frieden, 1959). The most marked changes in net protein accumulation were found in the mitochondrial and microsomal fractions. There is a shift during metamorphosis in the distribution of proteins from the soluble to the particulate fractions of the cell.

When the time-course of induction of the synthesis of some specific proteins involved in metamorphosis was studied, a lag period of 70–100 hr. after hormone administration was observed for most of them (Fig. 2). This lag period was usually followed by an abrupt increase in the activity of urea-cycle enzymes in the liver and hydrolases in the tail, as well as by the appearance of serum albumin and adult haemoglobin in the blood. It was therefore decided to pay special attention to synthesis and turnover of RNA during the first 100–150 hr. after the induction of metamorphosis.

### *Synthesis and turnover of RNA before and after induction of metamorphosis*

As nothing was known about the kinetics of synthesis of nuclear RNA and its transfer into the

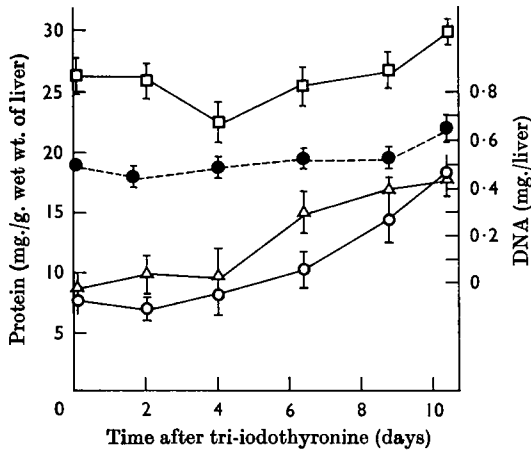


Fig. 1. Protein content of hepatic mitochondria ( $\Delta$ ), microsomes ( $\circ$ ) and cell sap ( $\square$ ) on the induction of metamorphosis in *Rana catesbeiana* tadpoles. Metamorphosis was induced by the injection of 0.5–1.0  $\mu$ g. of tri-iodothyronine in groups of 8–12 tadpoles. The values at zero time are for pre-metamorphic control animals. The total amount of DNA per liver is also shown ( $\bullet$ ); vertical bars denote the deviation from mean values.

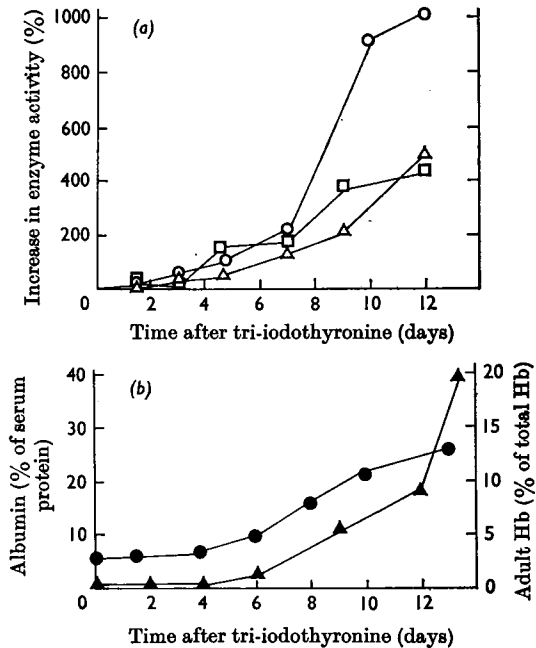


Fig. 2. Induction or accelerated synthesis of (a) hepatic and tail enzymes and (b) blood proteins in bullfrog tadpoles after the injection of tri-iodothyronine. The enzyme activities in control or non-induced tadpole tissues were: hepatic carbamoyl phosphate synthetase ( $\circ$ ),  $0.62 \pm 0.20$   $\mu$ -mole of citrulline formed/hr./mg. of protein; cytochrome oxidase in liver mitochondria ( $\square$ ),  $0.16 \pm 0.03$   $\mu$ mole of cytochrome c oxidized/min./mg. of protein; tail acid phosphatase ( $\Delta$ ),  $18 \pm 6$   $\mu$ g. of phosphate released/20 min./mg. of protein. The concentrations of serum albumin ( $\bullet$ ) and adult haemoglobin ( $\blacktriangle$ ) were calculated as percentages of total serum proteins and haemoglobin (Hb) respectively.

cytoplasm in anuran tadpoles, it became necessary to investigate this in pre-metamorphic bullfrog tadpoles before studying the effects of metamorphosis on RNA synthesis. Fig. 3 shows the time-course of the incorporation and accumulation of [ $^3$ H]uridine and [ $^{32}$ P]phosphate into nuclear and cytoplasmic RNA of tadpole liver. The pattern of incorporation is qualitatively similar to that described in mammalian liver (see Tata & Widnell, 1966), but the transfer of RNA from the nucleus into the cytoplasm is slower in the amphibian larval tissue. ([ $^{14}$ C]Orotic acid was also incorporated in a fashion similar to that of [ $^3$ H]uridine, but was not used as a routine to study RNA metabolism.) On the basis of the kinetics described in Fig. 3 the incorporation of [ $^3$ H]uridine was used as an index of the effect of induction of metamorphosis on the rate of synthesis of rapidly labelled nuclear RNA;  $^{32}$ P was used to follow the accumulation of newly synthesized RNA in the cytoplasm. The results, summarized in Fig. 4, show that the administration of tri-iodothyronine caused a relatively early increase in the specific radioactivity of rapidly labelled nuclear RNA; this value reached three to five times that in the uninduced controls at 48–72 hr. after hormone injection. At this time, no increase in inducible enzymes or total protein content was detected (see Figs. 1 and 2). The accelerated synthesis of RNA in the nucleus was followed at longer time-intervals after induction of metamor-

phosis by the appearance of cytoplasmic RNA of increasing specific activity (Fig. 4b). It was not possible to study the incorporation of radioactive precursors into RNA nor to follow RNA turnover beyond 5–6 days after tri-iodothyronine administration because of the rapid regression of several tissues, such as the tail, gut and gills, which sets in at about this time. A rapid tissue regression would be expected to alter drastically the pool sizes of RNA precursors, and thus explain the apparent decrease in specific activities of RNA in tadpoles that received the radioactive precursor 5 days or more after the hormone.

Only a part of the early increase in specific activity of nuclear and cytoplasmic RNA in metamorphosing animals could be ascribed to an increased rate of uptake of the radioactive precursor by the liver. Fig. 5 shows that the increase in total radioactivity taken up by the liver was

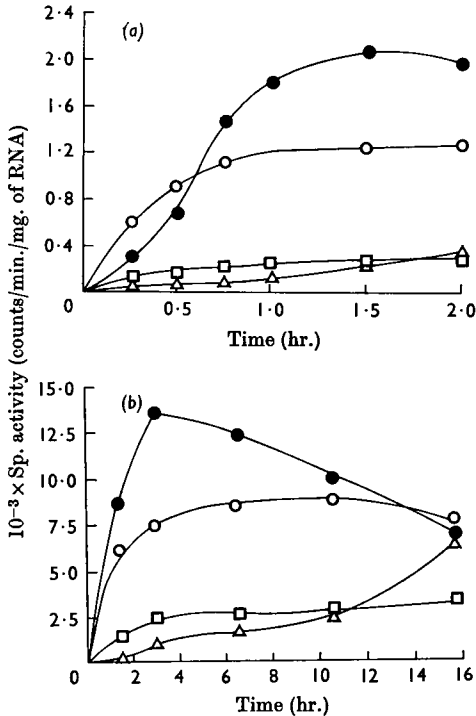


Fig. 3. Time-course of incorporation of (a)  $6\mu\text{C}$  of  $[^3\text{H}]$ -uridine and (b)  $11\mu\text{C}$  of  $[^{32}\text{P}]$ phosphate into nuclear and cytoplasmic RNA in the livers of pre-metamorphic bullfrog tadpoles. Nuclei (●), mitochondria (□), microsomes (Δ) and cell sap (○) were first separated and RNA was extracted from the subcellular fractions as described in the text.

accompanied by a marked fall in the fraction of acid-soluble radioactivity. The magnitude of these changes is such that it is reasonable to conclude that the increased specific activity of RNA reflects a true enhancement in the rate of its synthesis. Chemical determinations of the amount of RNA in the nucleus and cytoplasm did not reveal a rapid increase in the total RNA content of the tissue during the early phase of metamorphosis (Table 1). The virtual constancy of RNA content of the nucleus therefore suggests that an acceleration of RNA synthesis during metamorphosis is accompanied by an enhanced rate of transfer of RNA from the nucleus into the cytoplasm. The amount of ribosomal RNA in the cytoplasm had also increased by only 5–10% by the sixth day after induction of metamorphosis but by the twelfth day to 50–80% above controls. This result raises the possibility that the induction of metamorphosis accelerates initially a turnover of cytoplasmic RNA before any accumulation can be observed.

When the nature of the newly synthesized RNA

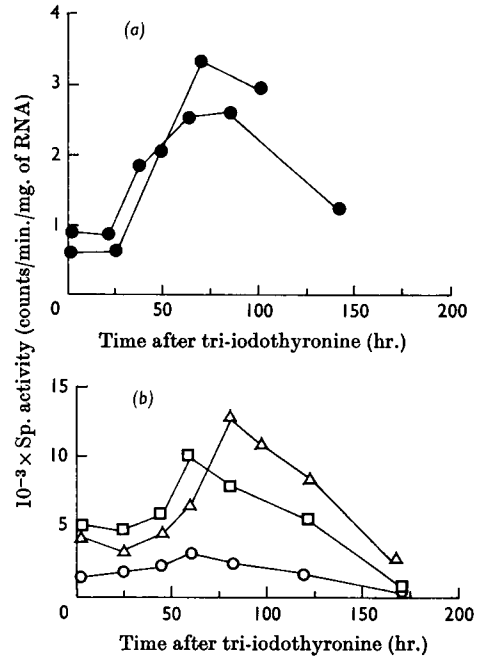


Fig. 4. Stimulation of the incorporation of (a)  $[^3\text{H}]$ uridine into rapidly labelled nuclear RNA (●) and (b)  $[^{32}\text{P}]$ phosphate into RNA from mitochondria (□), microsomes (Δ) and cell sap (○) from tadpole livers after the induction of metamorphosis by tri-iodothyronine. Groups of nine tadpoles were killed (a) 25 min. after  $10\mu\text{C}$  of  $[^3\text{H}]$ uridine or (b) 15.5 hr. after  $125\mu\text{C}$  of  $[^{32}\text{P}]$ phosphate. Values for nuclear RNA are from two different experiments.

was studied, little difference was found in the RNA recovered from the nucleus and ribosomes of metamorphosing and non-metamorphosing tadpoles (Table 2). The distribution of  $^{32}\text{P}$  in the bases of nuclear RNA was close to that of ribosomal RNA in both groups of animals, thus suggesting that much of the new RNA made upon the induction of metamorphosis was of the ribosomal type. A sucrose-density-gradient analysis of the RNA recovered from cytoplasmic ribosomes is shown in Fig. 6. There was considerable breakdown of tadpole liver RNA during extraction from both the cytoplasm and nucleus. This degradation was not prevented by polyvinyl sulphate, bentonite or macaloid, thus making it impossible to study the high-molecular-weight precursor RNA in the nucleus. It is for this reason that sucrose-density-gradient profiles of newly synthesized nuclear RNA are not presented in this paper. Although there was much degradation of cytoplasmic ribosomal RNA during its extraction, the differences observed between the preparations from metamorphosing

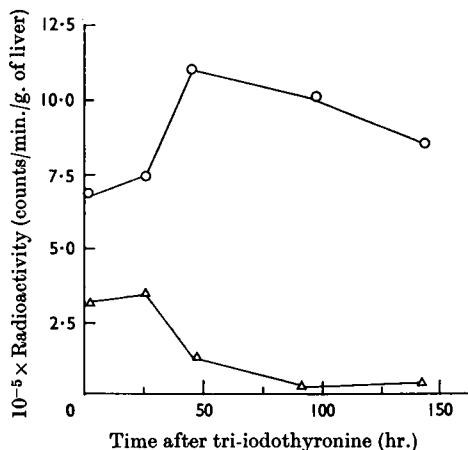


Fig. 5. Total radioactivity in tadpole liver (O) and in the fraction of the tissue homogenate soluble in 0.4N-HClO<sub>4</sub> (Δ), 25 min. after 10 μC of [<sup>3</sup>H]uridine in pre-metamorphic (zero time) and metamorphosing bullfrog tadpoles.

and non-metamorphosing animals were reproducible.

*Formation and distribution of ribosomes and RNA in the cytoplasm*

I have already demonstrated (Tata, 1965) that during the early stages of metamorphosis much of the newly formed cytoplasmic RNA was recoverable in monomeric ribosomes and polyribosomes. Further studies, illustrated in the sucrose-density-gradient profiles of Fig. 7, showed that induction of metamorphosis causes an increased amount of radioactive RNA to appear in the 78s monomers and 100s dimers as well as in the polysomes. The polysomal profiles obtained by detergent treatment of the mitochondria-free supernatant, rather than microsomes, were more reproducible, presumably because of the presence in the cell sap of a ribonuclease inhibitor (Blobel & Potter, 1966). It was not possible to demonstrate even at early stages of

Table 1. *Distribution of RNA in subcellular fractions of bullfrog tadpole liver before and after induction of metamorphosis*

The values ± S.D. were derived from a number of different experiments.

Time after tri-iodothyronine (days)	Metamorphic index (hind-limb/tail length ratio)	Fraction	RNA (mg./g. of liver)	RNA/DNA ratio	RNA/protein ratio
0	0-0.02	Nuclei	0.40 ± 0.11	0.35 ± 0.09	0.081 ± 0.034
		Microsomes	2.01 ± 0.31	—	0.108 ± 0.024
		Soluble	0.53 ± 0.14	—	0.019 ± 0.004
2.8	0-0.05	Nuclei	0.37 ± 0.10	0.38 ± 0.15	0.070 ± 0.031
		Microsomes	2.11 ± 0.26	—	0.109 ± 0.020
		Soluble	0.56 ± 0.07	—	0.015 ± 0.005
7.7	0.18-0.32	Nuclei	0.44 ± 0.08	0.32 ± 0.06	0.087 ± 0.026
		Microsomes	2.35 ± 0.22	—	0.118 ± 0.019
		Soluble	0.66 ± 0.16	—	0.018 ± 0.002

Table 2. *Distribution of <sup>32</sup>P in bases of nuclear and ribosomal RNA obtained from pre-metamorphic and metamorphosing tadpoles*

RNA was extracted for this purpose by a modified Schmidt-Thannhauser procedure (Tata & Widnell, 1966). Metamorphosis was induced by injection of 0.5 μg. of tri-iodothyronine to half of the 18 tadpoles 4.8 days before they were killed. All tadpoles were killed 9.5 hr. after an injection of 25.5 μC of [<sup>32</sup>P]phosphate. The recovery of the four nucleotides was in the range 90-95%. Ribosomal RNA was extracted from the pellet obtained after centrifuging a mitochondria-free supernatant treated with 0.25% sodium deoxycholate at 105000g for 70 min.

Tadpoles	RNA	Sp. activity (counts/min./μg. of P)	Percentage of <sup>32</sup> P recovered			
			AMP	GMP	CMP	UMP
Pre-metamorphic	Nuclear	110	20.9	32.8	24.0	22.1
	Ribosomal	55	18.7	34.0	24.6	19.2
Metamorphosis	Nuclear	466	21.6	29.5	22.4	24.6
	Ribosomal	143	20.3	33.2	23.7	20.6

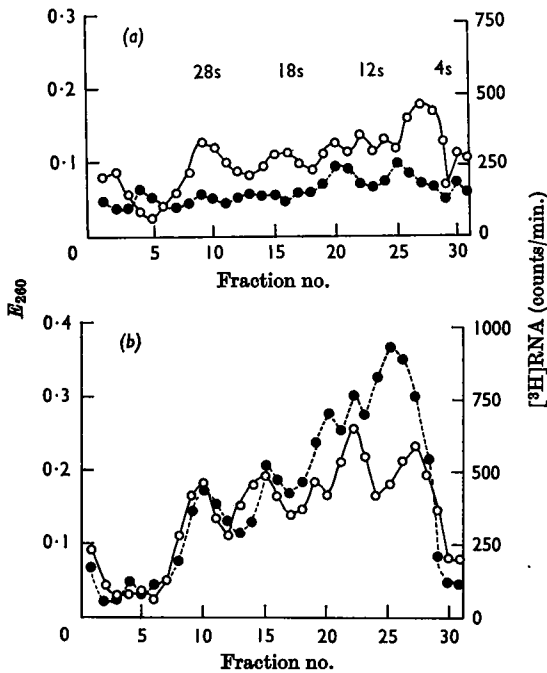


Fig. 6. Sucrose-density-gradient centrifugation analysis of liver microsomal RNA in (a) pre-metamorphic tadpoles and (b) tadpoles treated 7 days earlier with tri-iodothyronine. Fifteen animals were used in each group and  $10\mu\text{C}$  of [ $^3\text{H}$ ]uridine was administered 22.5 hr. before the livers were removed. RNA was extracted from microsomes with phenol-sodium dodecyl sulphate, as described in the text, and 100–200  $\mu\text{g}$ . of RNA in 1 ml. was layered over 29 ml. of a linear 5–20% sucrose gradient containing 50 mM-sodium acetate buffer, pH 5.1, and 0.1 M-NaCl in a Spinco SW 25.1 tube. The samples were centrifuged at 22 500 rev./min. for 16 hr. and 0.9 ml. fractions were removed from the bottom of the tube. The Figure denotes the total  $E_{260}$  (O) and radioactivity ( $\bullet$ ) in a 0.2 N-HClO<sub>4</sub> precipitate in each fraction. The numbers at the top of the Figure give the *S* values of the major component of RNA analysed under identical conditions.

metamorphosis and with shorter pulses of radioactive precursors that the effect of the hormone was restricted to the polysome fraction. It is likely therefore that newly formed m-RNA and ribosomes appeared together in the cytoplasm. At the same time there was during metamorphosis a marked increase in specific activity of the RNA in that region of the gradient immediately above the monomeric ribosome region. This would correspond to the ribosomal-precursor-particle region of the type detected in mammalian tissues. These particles in mammalian tissues have been shown to be a complex of m-RNA and one of the ribosomal sub-units of *S* 45–60s and their abundance reflects

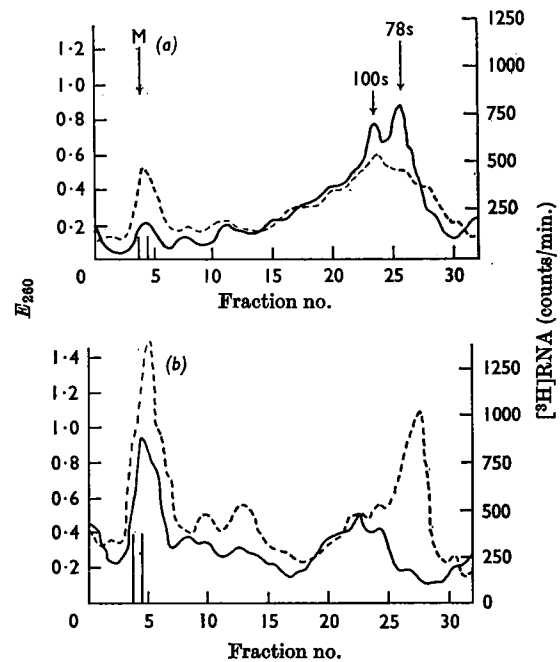


Fig. 7. Distribution of radioactive RNA in polysomal profiles from livers of (a) pre-metamorphic bullfrog tadpoles and (b) animals in which metamorphosis was induced 5–7 days earlier. RNA was labelled by the administration of  $10\mu\text{C}$  of [ $^3\text{H}$ ]uridine to each of the two groups of 12 tadpoles, 20.5 hr. before they were killed. Mitochondria-free supernatants were treated with 0.25% sodium deoxycholate and 2.0 ml. of the suspension was layered on top of 20 ml. of a linear 15–30% (w/v) sucrose gradient containing 10 mM-tris-HCl buffer, pH 7.6, 50 mM-KCl and 5 mM-MgCl<sub>2</sub> in a Spinco SW 25.1 tube. A layer of 4.5 ml. of 60% (w/v) sucrose, with the same buffer and salts, was layered at the bottom of the tube to trap the membrane-bound ribosomes at the interface of the 30% and 60% (w/v) sucrose and whose position is indicated by the arrow M. The preparations were centrifuged at 22 500 rev./min. for 2.5 hr. and 0.8–0.9 ml. fractions were collected from the bottom of the tube upwards. A quarter of each fraction was diluted with water to 3 ml. for measurement of extinction and the radioactivity determined in the remainder after precipitation with 5% trichloroacetic acid. The positions of the 78s monomeric ribosomes and 100s dimers are indicated by the downward pointing arrows (the sedimentation coefficients were determined on the isolated fractions in the analytical ultracentrifuge). —,  $E_{260}$ , ----, radioactivity.

the rate of transfer of m-RNA and r-RNA from the nucleus to the cytoplasm (Girard *et al.* 1965; Henshaw *et al.* 1965).

As already pointed out (Fig. 7 and Tata, 1965) a relatively higher proportion of newly formed polysomal RNA in metamorphosing tadpoles was more tightly bound to microsomal membranes than



Table 3. *Effect of inducing metamorphosis on the relative distribution of newly synthesized RNA in membrane-bound and free ribosomal fractions from liver mitochondria-free supernatants treated with different amounts of sodium deoxycholate*

Microsomal RNA was labelled by injecting 11.5  $\mu\text{C}$  of [ $^3\text{H}$ ]uridine per tadpole 22.5 hr. before they were killed. Each group comprised ten tadpoles and one group was treated with 0.7  $\mu\text{g}$ . of tri-iodothyronine 6 days before the experiment. Equal amounts of mitochondria-free supernatants were treated with different quantities of sodium deoxycholate, indicated by their final concentration, but the sucrose-density-gradient centrifugation was carried out in a Spinco no. 30 rotor as described by Tata (1965). Fractions (1 ml.) were then pooled, after determination of  $E_{260}$ , into the four fractions. The 'membrane-bound' ribosomal fraction is that which did not sediment through the 2.0 M-sucrose interface.

Tadpoles	Concn. of sodium deoxycholate (%)	Sp. activity (counts/min./ $E_{260}$ unit) and distribution of RNA in fractions (% in parentheses)			
		Membrane-bound ribosomes	Polysomes	Dimers+ monomers*	< 50s
Control	0.08	295 (4.7)	227 (21.5)	107 (56.6)	58 (12.0)
	0.16	418 (2.0)	254 (22.8)	92 (53.0)	41 (8.8)
	0.30	— (0)	238 (18.7)	122 (62.5)	73 (14.5)
6 days after tri-iodothyronine	0.08	875 (20.5)	430 (33.5)	492 (26.0)	196 (10.5)
	0.16	990 (12.8)	518 (35.1)	631 (32.9)	281 (15.1)
	0.30	1185 (1.5)	550 (25.8)	675 (29.0)	255 (11.9)

\* Also includes ribosomal precursor particles smaller than 78s monomers.

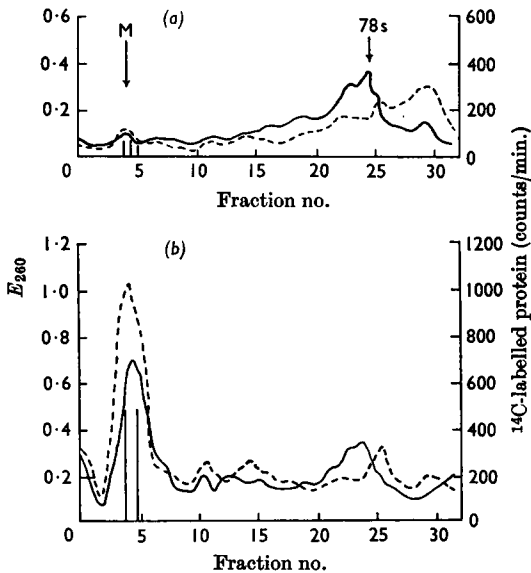


Fig. 8. Distribution of nascent protein synthesized *in vivo* on hepatic polysomes obtained from (a) pre-metamorphic bullfrog tadpoles and (b) animals in which metamorphosis had been induced 7.0 days before the preparations were made. Proteins were labelled by injecting 4  $\mu\text{C}$  of a mixture of  $^{14}\text{C}$ -labelled amino acids (algal-protein hydrolysate) 15 min. before the animals were killed. Polysome profiles were determined on mitochondria-free supernatant treated with 0.15% sodium deoxycholate. All other details were the same as in Fig. 7. —,  $E_{260}$ ; ----, radioactivity.

in controls. This feature is depicted in Table 3. It can be seen that hepatic ribosomes in pre-metamorphic tadpoles require much less deoxycholate for complete release from the membranes to which they are bound than do adult mammalian hepatic ribosomes. In metamorphosing animals two to three times as much detergent is required to achieve the same degree of dissociation.

The physiological significance of the fraction of tightly membrane-bound ribosomes became more obvious when the synthesis of proteins was studied *in vivo*. In these experiments polyribosomes were deliberately prepared at a low concentration of deoxycholate (0.15%) so that about 5–10% of ribosomes in control animals and 20–30% in metamorphosing tadpoles still remained attached to membranes (Fig. 8). When tadpoles were killed 15 min. after a pulse of radioactive amino acids, the bulk of nascent protein was recovered in the polysomes and in the membrane-attached ribosomes; the protein in the latter fraction had a higher specific activity than that associated with free polysomes. The overall specific activity of total microsomal protein was about twice as high as the controls in tadpoles treated 6 days earlier with the hormone. Much of this additional protein made in metamorphosing animals was associated with the small fraction of tightly membrane-bound particles. The change in the relative distribution of functionally active polysomes attached to membranes prompted me to look for possible ultrastructural

Table 4. *Properties of hepatic ribosomes from pre-metamorphic tadpoles and from animals 7.5 days after induction of metamorphosis*

Ribosomes were prepared from mitochondria-free supernatant, treated with 0.18% sodium deoxycholate for the pre-metamorphic animals and 0.38% for the metamorphosing animals, by centrifugation in a sucrose density gradient as described by Tata (1965). The fractions corresponding to the monomer and dimer peaks were pooled and ribosomes sedimented in the homogenizing medium by centrifuging at 105000g for 2 hr. The ribosomal pellet was suspended in 50 mM-tris-HCl buffer, pH 7.4, 0.1 M-KCl and 5 mM-MgCl<sub>2</sub> for determination of sedimentation coefficient or in 50 mM-sodium acetate buffer, pH 5.4, and 50 mM-KCl for extraction of RNA. Livers were pooled from 12 tadpoles for each group. Each value is the average of three or four determinations.

Tadpoles	$S_{20,w}$ of ribosomes* (s)	$S_{20,w}$ of ribosomal RNA (s)	RNA base composition (%)			
			AMP	GMP	CMP	UMP
Pre-metamorphic	78,100	16.9, 27.5	17.1	33.0	26.1	22.5
Metamorphosing	78,102	17.0, 27.3	16.8	33.5	25.6	23.3

\* Values for monomers and dimers.

alterations in the endoplasmic reticulum during induced metamorphosis.

#### *Electron and light microscopy*

Sections of the same livers as those used for determining polyribosomal distribution (Figs. 7 and 8 and Table 3) were examined by electron and light microscopy. Brief mention of the overall ultrastructural changes that occur in hepatic cells during induced metamorphosis has already been made (Tata, 1967a). Plates 1 and 2 illustrate the marked changes that take place in the form and extent of the rough endoplasmic reticulum and of polyribosome complexes. Before induction of metamorphosis, ribosomes in the liver cell cytoplasm were distributed in a more or less random manner and most were unattached or associated with relatively simple vesicular membranes (Plate 1c). Five days after administration of thyroid hormone nearly all the hepatic parenchymal cells had areas of cytoplasm with a much increased population density of ribosomes (Plate 2a); moreover the ribosomes were predominantly attached to complex lamellar membrane systems, comparable with the cisternae of rough endoplasmic reticulum in the liver cells of many adult mammalian species (see Palade, Siekevitz & Caro, 1962; Fawcett, 1964). It is noteworthy that this shift in the predominant pattern of endoplasmic reticulum and the association of ribosomes coincides with their relatively tighter attachment, as shown by the sucrose-density-gradient analysis (see Fig. 7 and Table 3), and with the shift in the synthesis of phospholipids from smooth to rough membranes (see Table 7). In grazing sections polyribosome complexes appearing as rings, spirals or chains were frequently identifiable (Plate 2b); these were much more numerous in liver sections from metamorphosing tadpoles than in the uninduced controls.

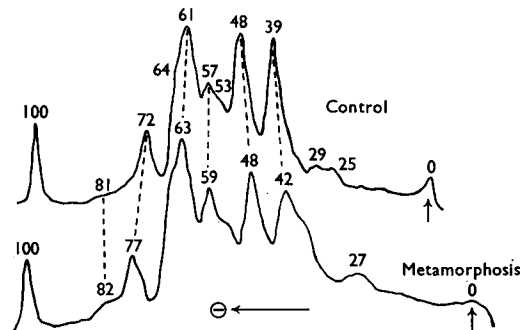
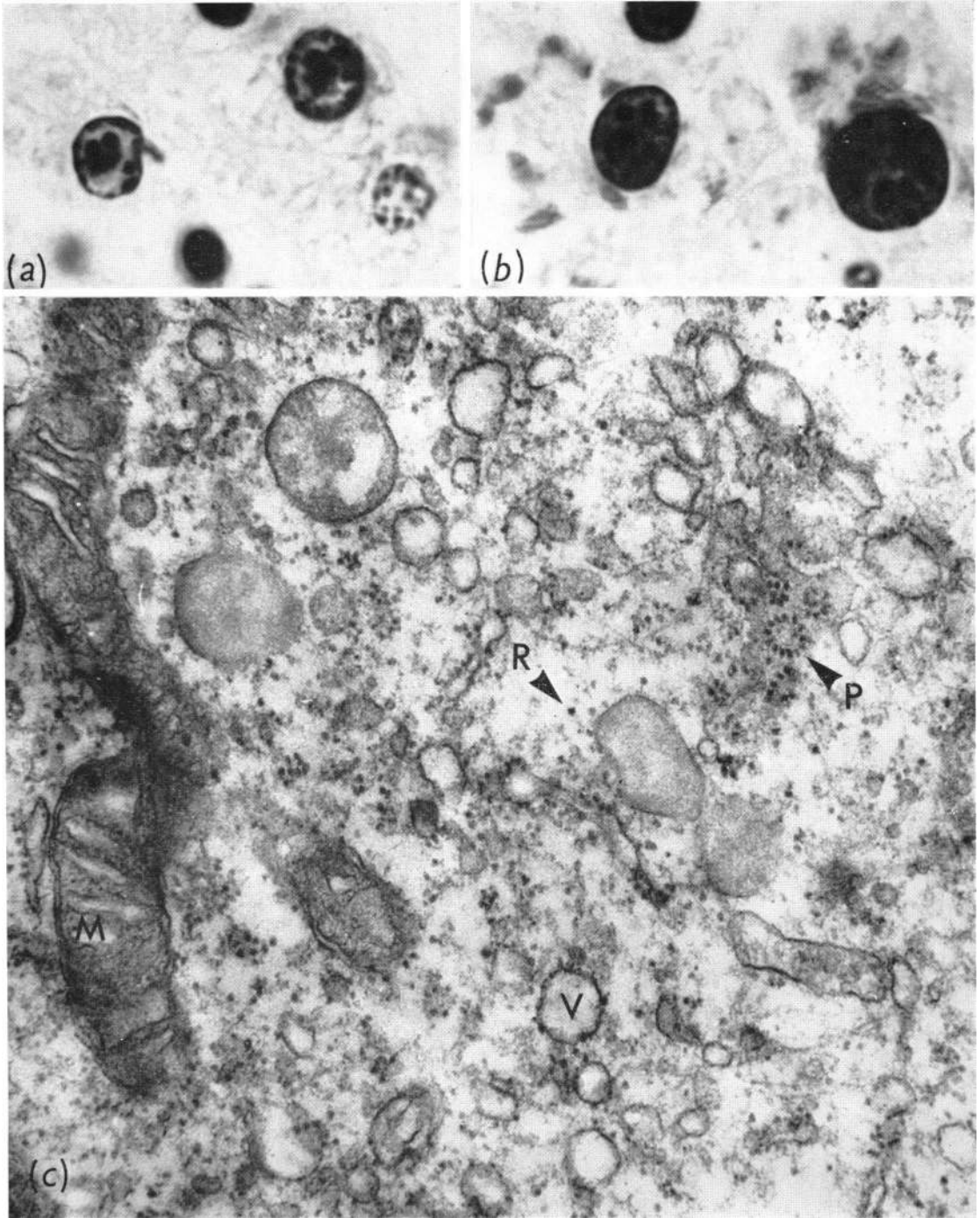


Fig. 9. Densitometric profiles of distribution of ribosomal structural proteins separated by polyacrylamide-gel electrophoresis at pH 4.3 and in the presence of 6M-urea. The proteins were extracted from ribosomal sub-units prepared from ribosomes from pre-metamorphic tadpoles (top curve) and tadpoles 7 days after the induction of metamorphosis (bottom curve). The ribosomes from the non-metamorphosing animals were obtained by centrifuging at 105000g for 75 min. in a 0.10% sodium deoxycholate-treated mitochondria-free supernatant. For metamorphosing animals 0.25% sodium deoxycholate was used for preparing the ribosomes. Other details are given in the text.

#### EXPLANATION OF PLATE 1

(a) and (b) Sections of bullfrog tadpole liver as seen by light microscopy, showing typical patterns of cytoplasmic basophilic material in a control (a) and during induced metamorphosis (b). In treated animals the larger clumps of basophilic material have a striated appearance. Toluidine blue stain was used. Magnification  $\times 1900$ . (c) Electron micrograph showing scattered distribution of ribosomes (R) in hepatic cell cytoplasm of a pre-metamorphic tadpole. A few ribosomes are associated with simple vesicular membranes (V). A well-defined annular polysome complex is shown at P. M, Mitochondrion. Magnification  $\times 60000$ .



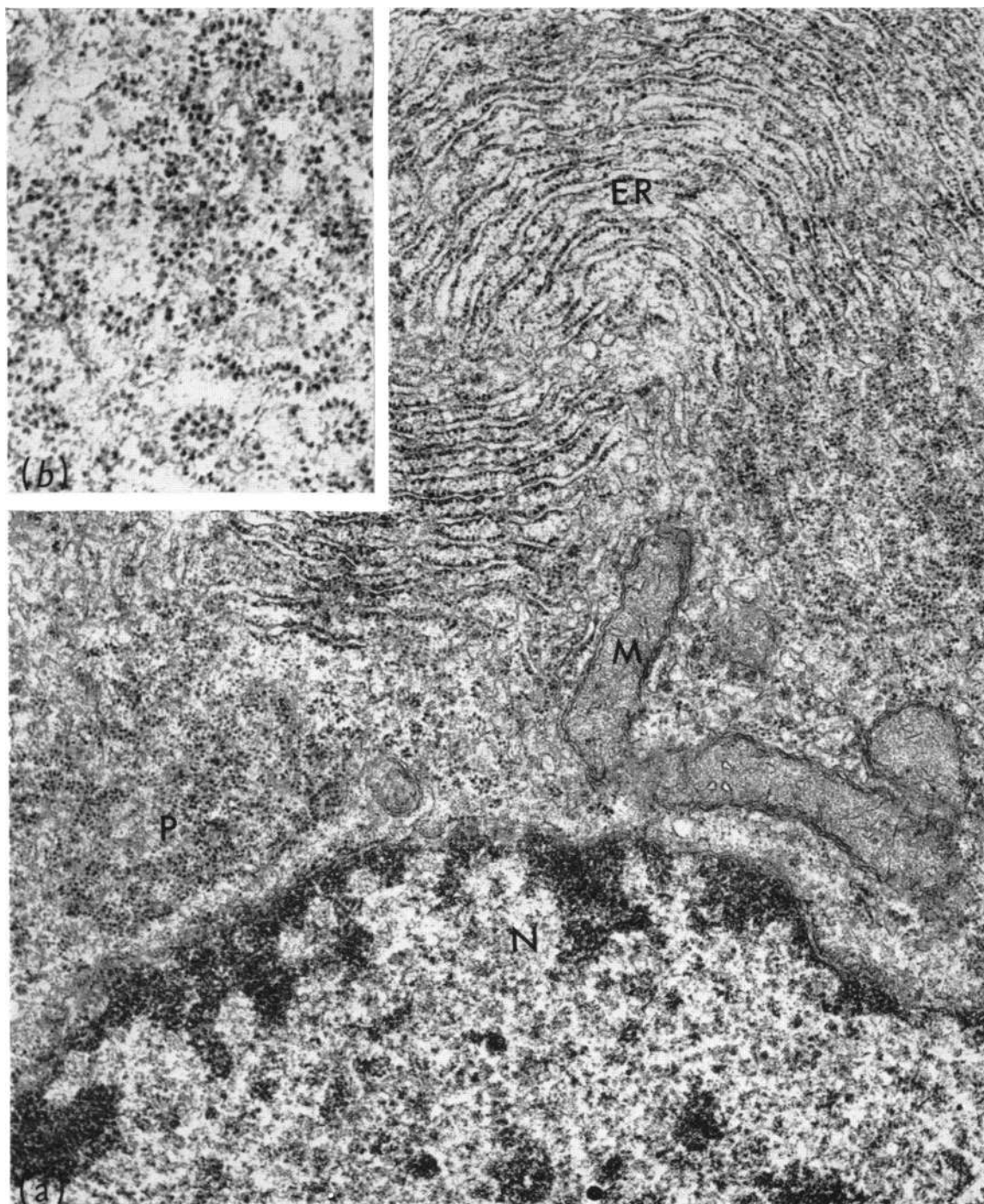


Table 5. Increase in the phospholipid content of particulate fractions of tadpole liver on the induction of metamorphosis

The values are averaged from three experiments, with seven or nine tadpoles in each group.

Fraction	Time after induction (days)	Phospholipid content (mg./g. of liver)	Phospholipid/protein ratio
Nuclei	0	0.38	0.114
	3.8	0.36	0.092
	6.8	0.48	0.101
Mitochondria	0	0.81	0.086
	3.8	0.98	0.079
	6.8	1.27	0.102
Microsomes	0	1.95	0.160
	3.8	2.11	0.165
	6.8	2.93	0.182

Search for other ultrastructural changes accompanying metamorphosis suggested that mitochondria may be more numerous in the liver cells of treated animals than in the pre-metamorphic controls; certainly they exhibited more complex profiles on section and contained more elaborate patterns of internal cristae. This is compatible with the recorded increase in total mitochondrial protein and specific activity of cytochrome oxidase. It would be interesting to correlate the increased rate of synthesis of ribosomal RNA with changes in nucleolar size or number, but no convincing change in nucleolar morphology was detected. Morphological evidence of altered nucleolar function should perhaps be looked for earlier than 5 days after administration of the hormone.

In paraffin sections an altered pattern of cytoplasmic basophilia was seen with the light-microscope after induction of metamorphosis (Plate 1a and 1b). Darkly staining and often striated cytoplasmic areas seemed to reflect the proliferation of rough endoplasmic reticulum shown by electron microscopy. Increased basophilia was present throughout the tissue; thus it is unlikely that the ultrastructural changes described here were due to fortuitous sampling of atypical areas.

#### EXPLANATION OF PLATE 2

(a) Electron micrograph of part of hepatic cell from a tadpole undergoing induced metamorphosis. The cytoplasm has a high density of ribosomes, of which many are associated with membranes in rough endoplasmic reticulum (ER). Polysomes are found in areas (P) where the section grazes the endoplasmic reticulum. N, Nucleus; M, mitochondrion. Magnification  $\times 32000$ . (b) Electron micrograph, at higher magnification, of hepatic cell cytoplasm in a metamorphosing tadpole, showing well-developed polysome complexes. Magnification  $\times 60000$ .

#### Composition of ribosomes and membrane proliferation

The marked morphological redistribution and turnover of ribosomes during metamorphosis raised two further questions: (a) Are the ribosomes formed before and after the induction of metamorphosis identical in their structure and composition? (b) Are the newly formed ribosomes during metamorphosis preferentially bound to newly formed membranes?

*Composition of ribosomes.* To answer the first question I studied the sedimentation coefficients of ribosomes and of the RNA extracted from them, the base composition of RNA and the profile of structural ribosomal proteins. Table 4 summarizes the data on size of the ribosomes and their RNA at two different stages of induced metamorphosis and it can be seen that there is no divergence from the values for ribosomes in non-metamorphosing tadpoles. A feature in all groups of animals was the greater abundance of 100s dimers than the 78s monomers. There were also no large differences in the gross ribosomal protein profiles in preparations from the different groups of tadpoles (Fig. 9). It should be noted that the firmer binding of ribosomes to membranes during metamorphosis has been exploited to enrich the preparations from metamorphosing animals in newly synthesized ribosomes formed during the early stages of the process (see the Experimental section). The profiles of basic ribosomal proteins in Fig. 9 show a lower number of protein bands than those observed by other workers in bacterial and mammalian ribosomes (Waller, 1964; Low & Wool, 1967). However, my extracts were prepared from ribosomal sub-units, which would eliminate contamination with nascent proteins that might remain attached to intact ribosomes.

*Rate of membrane phospholipid synthesis during*

*metamorphosis*. In answering the second question about the changes in distribution of ribosomes on membranes it became necessary to study the rate of proliferation of cytoplasmic membranes, especially those of the endoplasmic reticulum. Chemical analyses of the different subcellular particulate fractions showed that the microsomal fraction had a higher phospholipid content than mitochondria or nuclei (Table 5). When the microsomes were further fractionated into their various membranous constituents, the bulk of the microsomal

phospholipids was recovered in the smooth membrane fraction. This correlated well with the preponderance of smooth membrane profiles observed by electron microscopy of livers of non-metamorphosing animals (Plate 1).

Fig. 10 shows that the rate of labelling of phospholipids with  $^{32}\text{P}$  was higher in microsomes than in nuclei or mitochondria. Of the different microsomal fractions the phospholipids of the smooth membranes were labelled more rapidly than those of the rough membranes. The nature of phospholipids in the microsomal membranes was analysed by thin-layer chromatography, and the relative distribution of  $^{32}\text{P}$  in the different constituents, listed in Table 6, was in general found to be similar to that of hepatic microsomal phospholipids in mammals (Maganiello & Phillips, 1965; Dallner *et al.* 1966).

The rate of synthesis of phospholipids from all types of microsomal membranes was substantially elevated at about 6 days after the induction of metamorphosis. Table 7 shows this effect in experiments in which the phospholipids were labelled with  $^{14}\text{C}$ choline. As shown elsewhere (see Figs. 4b and 7), it is also at 6-7 days after the induction of metamorphosis that one notices the appearance of increasing amounts of newly made RNA and ribosomes in the cytoplasm. That the alterations during metamorphosis of the rates of synthesis of microsomal RNA and phospholipids were co-ordinated is shown by the experiment of Fig. 11, in which RNA and phospholipids were both labelled with  $^{32}\text{P}$ . Since the rates of labelling of microsomal RNA and phospholipids are different (see Figs. 4b and 10) the duration of pulse of  $^{32}\text{P}$ -phosphate was chosen to be intermediate to that for maximal labelling of the two components.

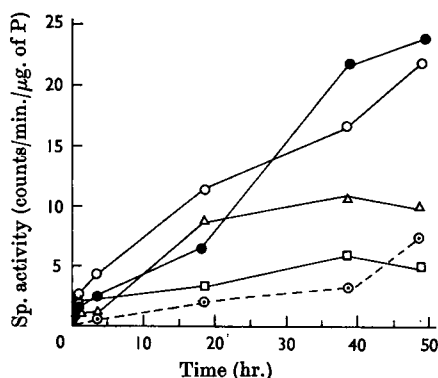


Fig. 10. Time-course of incorporation of  $^{32}\text{P}$ phosphate into phospholipids of nuclei ( $\square$ ), mitochondria ( $\Delta$ ) and the smooth ( $\circ$ ) and rough ( $\bullet$ ) microsomal membrane fractions of pre-metamorphic tadpole liver.  $^{32}\text{P}$ Phosphate ( $7.0\ \mu\text{C}$ ) was injected at different times before groups of eight tadpoles were killed per time-point and the subcellular fractionation and phospholipid extraction were carried out as described in the text. For comparison, the specific radioactivity of total microsomal RNA obtained from the same animals is also shown ( $\circ$ ).

Table 6. *Distribution of  $^{32}\text{P}$  in hepatic microsomal phospholipids of pre-metamorphic and metamorphosing tadpoles*

Groups of ten tadpoles were killed 15.5 hr. after an injection of  $8.0\ \mu\text{C}$  of  $^{32}\text{P}$ phosphate. Metamorphosis was induced with  $0.8\ \mu\text{g}$ . of tri-iodothyronine at the times indicated. Results (average of four determinations) are expressed as percentages of total microsomal phospholipid  $^{32}\text{P}$  recovered in each fraction.

Phospholipid	Days after induction	% of total phospholipid $^{32}\text{P}$			
		0	2.7	5.0	7.0
Lysolecithin	...	4.9	5.0	3.3	4.4
Sphingomyelin	...	5.7	2.1	1.8	1.8
Phosphatidylcholine	...	35.7	44.1	39.8	44.7
Phosphatidylinoside	...	2.1	1.8	2.0	1.5
Phosphatidylserine	...	15.2	13.2	13.9	16.7
Phosphatidylethanolamine	...	23.5	30.1	33.0	27.2
Phosphatidic acid	...	0.6	2.4	0.8	2.1
Unidentified*	...	7.8	1.6	5.5	2.5

\* Includes material remaining at the origin and migrating to the front of the thin-layer chromatograms.

Table 7. Incorporation of [ $^{14}\text{C}$ ]choline into the phospholipids of smooth and rough membranes of tadpole hepatic microsomes before and after the induction of metamorphosis

Membrane phospholipids were labelled with  $0.6\ \mu\text{C}$  of [ $^{14}\text{C}$ ]choline chloride and tadpoles killed 2.2 hr. after its administration. Smooth and rough membranes were separated and phospholipids extracted from them as described in the text.

Time after induction (days)	Microsomal membranes	Phospholipid (mg./g. of liver)	Sp. activity (counts/min./mg. of phospholipid)	
0	Smooth	1.95	438	
	Rough	light	0.68	520
		heavy	0.31	810
3.0	Smooth	1.79	798	
	Rough	light	0.98	915
		heavy	0.27	1170
6.1	Smooth	2.30	724	
	Rough	light	0.92	1033
		heavy	0.52	1500

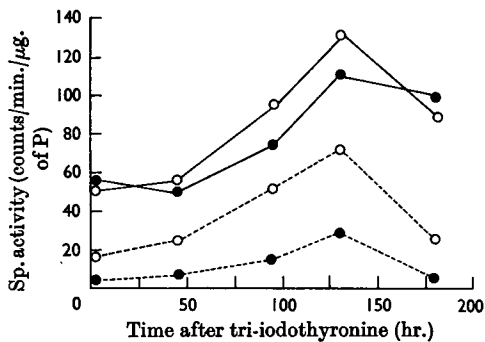


Fig. 11. Effect of induction of metamorphosis on the incorporation of [ $^{32}\text{P}$ ]phosphate into RNA and phospholipids of microsomal smooth and rough membranes of the bullfrog liver. Groups of ten tadpoles (for each time-interval) were injected with  $12\ \mu\text{C}$  of [ $^{32}\text{P}$ ]phosphate 45 min. before they were killed. Other details are given in the text. ○—○, Smooth membrane phospholipids; ●—●, rough membrane phospholipids; ○—○, smooth membrane RNA; ●—●, rough membrane RNA.

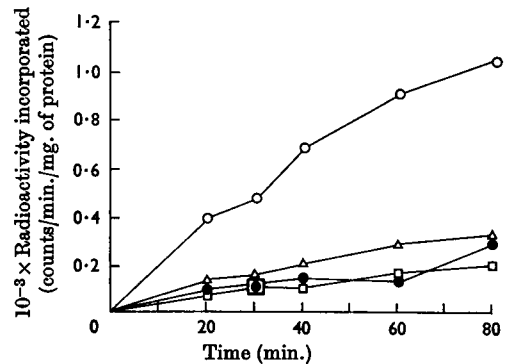


Fig. 12. Time-course of incorporation of amino acids into protein in liver nuclei (□), mitochondria (△), microsomes (○) and cell sap (●) in pre-metamorphic bullfrog tadpoles. Groups of nine tadpoles were killed at different times after injection of  $1.0\ \mu\text{C}$  of a mixture of  $^{14}\text{C}$ -labelled amino acids (algal-protein hydrolysate). Other details are given in the text.

#### Incorporation of amino acids into protein during induced metamorphosis

The final question was to determine how the changes brought about at the initiation of metamorphosis in the rates of ribosome and membrane synthesis were correlated with the protein-synthesizing capacity of the tissue. Fig. 12 shows the time-course of incorporation *in vivo* of labelled amino acids into proteins of all the major sub-cellular fractions of non-metamorphosing tadpole livers. As would be expected, microsomal protein was most heavily labelled at all pulse periods of  $^{14}\text{C}$ -labelled amino acids; the radioactive protein

appeared very slowly in the cell-sap fraction but increased as that in the microsomal fraction reached a plateau or decreased. The rate at which this process occurred in larval livers was considerably lower than that reported for adult mammalian livers (see Korner, 1964).

In studying how induction of metamorphosis would affect the rate of protein synthesis, attention was focused on the microsomal fraction, which was subjected to further subfractionation. Fig. 13 summarizes the results of such studies and shows that there was stimulation of all the microsomal subfractions between the third and fifth days after the hormone was administered. The reversal of

this stimulation after the seventh or eighth day may be, as with RNA synthesis (see Fig. 4), a consequence of expansion of amino acid pools caused by regression of some tissues. It is noteworthy that the specific activity of proteins in the smooth membrane fraction was nearly as high as in the light rough membrane fraction but not as high as in the heavy rough membrane fraction. However, unlike the finding in adult mammalian tissues, the bulk of microsomal membranes of tadpole liver

were of the smooth type and made up nearly 70% of total microsomal protein (Table 8). During metamorphosis, there was a gradual shift in the RNA content from smooth membranes to light rough membranes at first, and then to the heavier fractions.

## DISCUSSION

Although metamorphosis does not occur spontaneously in bullfrog tadpoles for 2-3 years, several enzymes and proteins associated with the process were induced within a few days after the administration of thyroid hormone. Many of the proteins and enzymes are induced in the liver and it has been previously shown for serum albumin and carbamoyl phosphate synthetase that administration of thyroxine caused synthesis to take place in the liver (Bennett & Frieden, 1962; Tatibana & Cohen, 1964). Under the conditions that I have used, there was a lag period of about 4 days after the injection of tri-iodothyronine before the detection of any induced protein synthesis. It is during this period that two important sequences of cellular responses were observed. After tri-iodothyronine administration there was relatively early stimulation (within 2 days) of the rate of nuclear and cytoplasmic RNA synthesis; this was followed by a turnover and redistribution of ribosomes. The latter process was accompanied by an accelerated synthesis but altered pattern of cytoplasmic membranes, which continued together with an increasing rate of enzyme and protein synthesis.

The time-course studies shown in Fig. 3 indicate that part of the 'rapidly labelled' nuclear RNA is gradually transferred into the cytoplasmic particulate fraction whereas transfer RNA of high specific activity appeared relatively early in the cytoplasm.

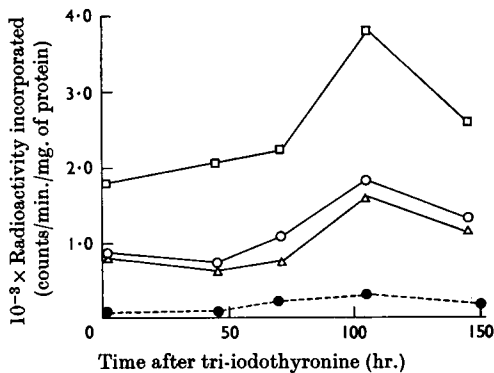


Fig. 13. Incorporation of  $^{14}\text{C}$ -labelled amino acids into proteins of hepatic submicrosomal fractions of bullfrog tadpoles as a function of time after the induction of metamorphosis with  $0.6\ \mu\text{g.}$  of tri-iodothyronine. A mixture of  $^{14}\text{C}$ -labelled amino acids ( $1.0\ \mu\text{C}$ ) was injected 50 min. before the tadpoles were killed. Smooth membranes ( $\Delta$ ), light rough membranes ( $\circ$ ), heavy rough membranes ( $\square$ ) and 'free' polysomes ( $\bullet$ ) were prepared from mitochondria-free supernatant by the technique of ultracentrifugation and without the use of detergents. Each point is the average of four determinations on material obtained from livers pooled from 12 tadpoles.

Table 8. Chemical composition of four fractions of the endoplasmic reticulum from livers of pre-metamorphic and metamorphosing tadpoles

These values were averaged from six different experiments in which groups of 8-12 tadpoles were used.

Time after induction (days)	Microsomal fraction	Composition (mg./g. equiv. of liver)			
		Protein	RNA	Phospholipid	
0	Smooth membranes	7.52	0.13	2.18	
	Rough membranes	light	2.30	0.27	0.35
		heavy	0.67	0.20	0.15
	'Free' polysomes	1.28	0.61	0.03	
3.7	Smooth membranes	7.35	0.18	2.25	
	Rough membranes	light	2.77	0.38	0.52
		heavy	0.79	0.23	0.24
	'Free' polysomes	0.81	0.32	0.03	
7.0	Smooth membranes	8.37	0.22	2.67	
	Rough membranes	light	2.75	0.45	0.54
		heavy	1.22	0.48	0.34
	'Free' polysomes	0.88	0.52	0.05	



This pattern of labelling is similar to, but slower than, that observed in mammalian liver (Tata & Widnell, 1966). The first major response of larval hepatocytes to the administration of thyroid hormone was a marked stimulation in the rate of synthesis of rapidly labelled nuclear RNA (Fig. 4a). When long pulses of [ $^{32}\text{P}$ ]phosphate were used, the effect of induction of metamorphosis was to enhance the labelling of all fractions of cytoplasmic RNA, an effect quite marked at 3–4 days after the administration of the hormone. [The reason why Nakagawa, Kim & Cohen (1967) found a higher specific activity of rapidly labelled RNA in microsomes rather than nuclei is not clear, but is likely to be due to nuclear breakage during homogenization of the liver.]

Because of its extensive degradation, attempts to characterize the nature of the newly synthesized RNA in the nucleus and cytoplasm on the induction of metamorphosis were unsuccessful. Degradation was not appreciably decreased by bentonite, macaloid or polyvinyl sulphate, whereas high-molecular-weight RNA (30–50s) could be extracted with little degradation from rat liver nuclei processed in the same way and at the same time. During the preparation of this paper Nakagawa & Cohen (1967) also reported extensive degradation of RNA extracted from whole livers of bullfrog tadpoles both before and after induction of metamorphosis. However, in spite of this breakdown, these authors conclude that the labelling patterns on sucrose density gradients (as well as  $^{32}\text{P}$  distribution in the RNA bases of whole liver) prove a selective induction by thyroxine of a 6–10s m-RNA needed for the synthesis of enzymes associated with metamorphosis. My data do not permit such a conclusion, although the rate of m-RNA synthesis may be accelerated during metamorphosis. The very slight shift in the pattern of  $^{32}\text{P}$  distribution in the bases of nuclear RNA during the early phase of metamorphosis (Table 4), in spite of a three- to four-fold increase in specific activity, suggests that much of the RNA made in the nucleus at this stage is of the ribosomal type. This conclusion has been confirmed in this Laboratory by a DNA-RNA hybridization analysis of rapidly labelled nuclear RNA. It demonstrates clearly that small changes in m-RNA synthesis during metamorphosis are accompanied by a very substantial acceleration of the labelling of r-RNA (G. R. Wyatt, C. C. Gonzalez & J. R. Tata, unpublished work). It is therefore very likely that a large part of the radioactivity recovered in the region of 6–10s is derived from a degradation of newly synthesized ribosomal RNA (presumably from the rapidly labelled 30–45s precursor molecules).

Perhaps a better index of an early effect on m-RNA turnover at the onset of metamorphosis

is the change observed in cytoplasmic polysome profiles (Figs. 7 and 8 and Table 3). The ratio of polysomes to monomers and dimers increased by the fourth to seventh days after the induction of metamorphosis, thus coinciding with additional protein synthesis. There was also a shift to larger polyribosomal aggregates during metamorphosis, which suggests a higher average size of new m-RNA or a denser packing of ribosomes on m-RNA during this process. However, from the increase in specific radioactivity in the 78s and 100s particles, it seems that the shifts in polysomal profiles were accompanied by the appearance in the cytoplasm of newly formed ribosomes. Such a phenomenon is compatible with the current concepts of a nuclear-cytoplasmic transport of m-RNA as a complex with a ribonucleoprotein ribosomal precursor particle (Girard *et al.* 1965; Henshaw *et al.* 1965; McConkey & Hopkins, 1965). In this connexion, the marked increase in specific activity of RNA from metamorphosing tadpoles in the 50–60s region of the polysomal profile in Fig. 7 should be noted. This fraction might represent a particle of the type shown to be a complex of m-RNA with the 30s ribosomal sub-unit and considered to be the precursor of polysomes (Girard *et al.* 1965; Henshaw, Revel & Hiatt, 1965; McConkey & Hopkins, 1965). Finkel, Henshaw & Hiatt (1966) found greatly increased specific activities of hepatic RNA in the precursor particles in adrenalectomized animals just before the induction by cortisol of adaptive hepatic enzymes could be detected. (It should be added, however, that my analytical system was not designed to detect polysomal precursors and patterns of the type shown in Fig. 4 were not observed repeatedly.)

The relatively slight net accumulation of RNA (Table 1), despite the increases in specific activity of both nuclear and microsomal RNA, as well as the increase in the average size of polysomes, suggested a turnover of microsomal RNA at the initial stages of metamorphosis. To test this possibility further, RNA turnover was studied by a technique of double-labelling. It was found by labelling RNA with [ $^3\text{H}$ ]uridine that the rate of loss of labelled ribosomal RNA was very slow, with a half-life of 7–8 days (this value is higher than that of 4–5 days for rat liver obtained for ribosomal RNA by Loeb, Howell & Tomkins, 1965). When metamorphosis was induced in tadpoles in which RNA has been labelled with [ $^3\text{H}$ ]uridine 8 days before receiving the hormone, and then followed by a 21 hr. pulse of [ $^{14}\text{C}$ ]orotic acid at different times after the hormone, the results summarized in Table 9 were obtained. The administration of triiodothyronine accelerated the breakdown of RNA labelled with  $^3\text{H}$  before metamorphosis, and at the same time incorporation of  $^{14}\text{C}$  into new RNA

Table 9. *Double-labelling of ribosomal RNA demonstrating that both breakdown and synthesis of RNA are accelerated at the onset of metamorphosis*

A batch of 64 tadpoles was divided into eight groups of eight each. Each tadpole received  $12\mu\text{C}$  of [ $^3\text{H}$ ]uridine 8 days before metamorphosis was induced (day 0). Two groups (one control, one induced) were then given  $2\mu\text{C}$  of [ $^{14}\text{C}$ ]orotic acid on days 0, 2, 4 and 6 and killed 24 hr. later. Ribosomes were prepared from mitochondria-free supernatant treated with 0.4% sodium deoxycholate and RNA was extracted by the modified Schmidt-Thannhauser method (Tata & Widnell, 1966).

Day on which [ $^{14}\text{C}$ ]orotic acid administered	Metamorphosis induced	Sp. activity (counts/min./mg. of RNA)		
		$^3\text{H}$	$^{14}\text{C}$	$^3\text{H}/^{14}\text{C}$ ratio
0	—	17350	4600	3.77
	+	14900	5230	2.85
2	—	15765	5680	2.78
	+	8010	7825	1.02
4	—	15500	3650	4.24
	+	6360	11300	0.56
6	—	12870	4100	3.13
	+	5085	9420	0.54

proceeded at a more rapid rate. Such double-labelling experiments had to be restricted to the early stages of metamorphosis: (a) because of the complications in precursor pool sizes that would arise from an eventual regression of some tissues and (b) because of marked accumulation of RNA 8–9 days after hormone administration, as shown previously by Finamore & Frieden (1960).

It has been suggested that, although membrane-free polysomes actively incorporate amino acids into protein *in vitro*, it is the fraction of ribosomes attached to cytoplasmic membranes that is most actively engaged in protein synthesis in the intact cell (Henshaw *et al.* 1963; Sabatini, Tashiro & Palade, 1966). The high yield of nascent protein in the relatively small fraction of membrane-bound ribosomes confirms the importance of the attachment of these particles to endoplasmic reticulum membranes during metamorphosis (Fig. 5). This is further emphasized by (a) the shift in the distribution of ribosomes from a relatively loose association with membranes to a firmer binding (Table 3 and Figs. 7b and 8b) and (b) the low specific activity of nascent protein associated with 'free' polysomes compared with those in the heavy rough membranes (Fig. 12). The shift in the distribution pattern of ribosomes bound to membranes of the endoplasmic reticulum was unequivocally confirmed by electron and light microscopy (Plates 1 and 2). It should be noted in considering a turnover, as against a simple accumulation of ribosomes at the initial stages of metamorphosis, that the type and distribution of membranes to which ribosomes are attached was also altered as development proceeded. Concomitant proliferation of ribosomes and membranes has also been observed in the developing

embryonic or neonatal liver of the rat (Dallner *et al.* 1966), in human embryonic liver (Zamboni, 1965) and in the androgen-dependent maturation of seminal vesicles in the mouse (Szirmai & van der Linde, 1965).

A change in the nature of ribosome-membrane interaction at the onset of metamorphosis could not be ascribed to any gross alterations of the physicochemical characteristics of newly formed ribosomes or their RNA and protein components (Table 4 and Fig. 9). Mutolo, Giudice, Hopps & Donatuti (1967) have also failed to discern any changes in structural ribosomal proteins during the development of the sea-urchin embryo. It was also not possible to discern any major shift in the pattern of labelling of phospholipids of microsomal membranes during metamorphosis. On the other hand, the increased rate of formation and turnover of ribosomes after induction is co-ordinated with that of the synthesis of membrane phospholipids as well as with the protein-synthesizing capacity of the microsomes. What is particularly interesting is that the newly formed ribosomes and membrane phospholipids tend to accumulate in the same sub-fraction of microsomes, i.e. the heavy rough membranes (Fig. 12 and Table 8). This raises the possibility that polysomes and membranes to which they are bound may be generated as a unit with a very low level of exchange between membranes and ribosomes formed at different stages of development. The findings of Loeb *et al.* (1965) for ribosomes and of Omura, Siekevitz & Palade (1967) for membrane proteins and lipids that the two constituents of rat liver are turned over at almost the same rate (half-life 4–5 days) may be quite important in this context. However, a

Table 10. *Lack of effect of thyroid hormones on the rate of synthesis of nuclear and microsomal RNA in neotenic amphibia (Necturus and axolotl)*

The animals were given acute or chronic treatment with thyroid hormones in different combinations before a short (2-3 hr.) or long (16-18 hr.) pulse of [<sup>32</sup>P]phosphate. The amount of [<sup>32</sup>P]phosphate used was 6-10 μ<sub>c</sub> for bullfrog tadpoles, 60-80 μ<sub>c</sub> for *Necturus* and 10 μ<sub>c</sub> for axolotl larvae. Acute treatment involved a single injection of 20 μg. of 3,3',5-tri-iodo-L-thyronine or L-thyroxine to each *Necturus* of 200-300 g. and 2.5 μg. of 3,3',5-tri-iodo-L-thyronine or 5 μg. of L-thyroxine per axolotl of 15 g. For chronic treatment the same amount of hormone was injected repeatedly. The days before killing when hormone administration was made are denoted in parentheses. Other conditions were as described in the text. T<sub>3</sub>, 3,3',5-Tri-iodo-L-thyronine; T<sub>4</sub>, L-thyroxine.

Species	Hormone administered	Type of treatment	[ <sup>32</sup> P]Phosphate pulse (hr.)	Sp. activity of RNA (counts/min./mg. of P)	
				Nuclear	Microsomal
Bullfrog tadpoles	—	—	3.0	10800	845
	—	—	18.0	6600	4530
	T <sub>3</sub>	Acute (4 days)	3.0	38350	1100
			18.0	30480	12700
	T <sub>4</sub>	Acute (4 days)	3.3	26125	1420
			17.5	15800	8100
<i>Necturus</i>	—	—	3.0	18950	570
	—	—	18.0	13030	2100
	T <sub>3</sub>	Acute (2 days)	3.0	12550	255
		Acute (6 days)	18.0	11600	1365
	T <sub>3</sub>	Chronic (1, 4 and 7 days)	12.5	11600	280
			16.7	13450	1400
	T <sub>4</sub>	Chronic (2, 5, 9, 12 and 15 days)	17.0	14600	2720
Axolotl	—	—	2.6	5250	160
	—	—	16.5	6700	495
	T <sub>3</sub>	Acute (2 days)	2.6	7015	—
		Acute (4 days)	18.5	—	630
	T <sub>4</sub>	Chronic (1, 3, 7 and 11 days)	16.5	—	380

convincing proof that ribosomes and membranes turn over at the same rate would need a single experiment in which they were both compared.

I was unable to extend the studies of labelling of macromolecules *in vivo* to measuring the rates of RNA and protein synthesis *in vitro* by nuclei and microsomes isolated at different stages of metamorphosis. The irreproducibility of results of determinations of RNA polymerase and amino acid incorporation by the subcellular particles was most likely due to a high activity of ribonuclease, which was not suppressed by polyvinyl sulphate. Nakagawa & Cohen (1967) have also reported a high ribonuclease activity in bullfrog tadpole liver, which was particularly active in releasing uracil residues. The RNA polymerase assay was also vitiated by a very active homopolymer formation when [<sup>14</sup>C]ATP was used as the substrate. By using isolated chromatin from tadpole liver and a bacterial RNA polymerase, Kim & Cohen (1966) were able to show that thyroxine enhanced the template activity of the chromatin.

A question that had to be answered at the early stages of this work was how far the effects on RNA

synthesis are specific with respect to the process of embryonic development and how far they merely represent non-specific response of the liver to hormone administration. The effect of thyroid hormone on a totally neotenic amphibian, the mud-puppy (*Necturus*), and a partially neotenic one, the axolotl, in which hormone administration did not induce metamorphosis, were compared. The results summarized in Table 10 show clearly that a variety of procedures of treatment of these non-metamorphosing amphibia with thyroid hormones failed to produce any change in the rate of RNA synthesis. Another facet of the question of specificity was to determine whether hormone-induced stimulation of RNA synthesis in bullfrog tadpoles was essential for the induction of the enzymes and proteins associated with metamorphosis. Attempts to demonstrate this dependence with the inhibitor actinomycin D were only partly successful because the range of doses of the inhibitor that were effective but not lethal was very narrow. A partial suppression by actinomycin D of the enhanced rate of synthesis of RNA by thyroxine in tadpole liver has, however, been reported (Nakagawa *et al.* 1967),

and a virtually total dependence of the metamorphic process on new RNA and protein synthesis has been shown for the tadpole tail (Weber, 1965; Tata, 1966b; Eeckhout, 1966).

Although thyroid hormones are among the slowest-acting of all developmental hormones in vertebrates (see Tata, 1966a), the lag period of nearly 40hr. for a stimulation of nuclear RNA synthesis is too long to suggest a direct action of thyroid hormone on RNA synthesis. Indeed the localization of the site of action is a major unsolved problem in our understanding of how hormones act. On the other hand, the type and sequences of cellular responses provoked by thyroid hormone at the onset of metamorphosis fall into a general pattern observed in many hormone-dependent growth and developmental systems (Tata, 1966a, 1967a,b). In particular, the simultaneous control of ribosome formation, cytoplasmic membrane proliferation and protein synthesis has now been shown in four distinct tissues in which different hormones were used to induce growth and maturation (Tata, 1967b; P. R. Kerkof & J. R. Tata, unpublished work). It seems that such a co-ordinated regulation of the synthesis of macromolecules and cell structures is in itself not the specific or initial action of hormones. It is more likely that the hormone triggers off a chain of unknown but specific reactions according to a set programme depending on the nature and stage of development of different systems, and this in turn provokes a sequence of fundamental but non-specific responses observed in all growing cells.

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