The Response of the Small Intestine to Vitamin D

ISOLATION AND PROPERTIES OF CHICK INTESTINAL POLYRIBOSOMES

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1. Undegraded polyribosome preparations may be obtained from chick intestinal mucosa if ribonuclease activity is strictly controlled. This is best achieved by homogenization of the mucosa directly in rat liver cell-sap. 2. The extent of amino acid incorporation by chick intestinal polyribosomes is greatly influenced by the source of the cell-sap. Sephadex-treated intestinal cell-sap caused impaired incorporation and release of completed polypeptide chains, whereas Sephadex-treated rat liver cell-sap promoted the polymerization of up to 90 amino acids per ribosome. Under optimum conditions 30–35% of the nascent polypeptide chains are completed and released. 3. The preparation of an antiserum against the calcium-binding protein formed in response to vitamin D is described. It is shown that the antiserum is highly specific for calcium-binding protein. 4. This antiserum was used to investigate the ability of chick intestinal polyribosomes to synthesize calcium-binding protein. Only polyribosomes from chicks receiving vitamin D have the ability to synthesize calcium-binding protein. Moreover, the product formed *in vitro* has the same electrophoretic mobility as calcium-binding protein synthesized *in vivo*. 5. It is concluded that one of the main functions of vitamin D in the small intestine is to induce the synthesis *de novo* of calcium-binding protein.

Knowledge of the mode of action of vitamin D has increased rapidly in the last decade, in terms of both the metabolic interconversions that occur and the response of the target cells to the active metabolites. It is well established that cholecalciferol must first be converted into the metabolites 25-hydroxycholecalciferol (Blunt *et al.*, 1968) and 1,25-dihydroxycholecalciferol (Lawson *et al.*, 1971) before calcium absorption across the small intestine is enhanced.

The effect of the active metabolites of vitamin D on the target cells, especially the intestinal cell, is well understood at the cellular level. Thus administration of cholecalciferol to a rachitic chick results in a stimulation of calcium absorption by the small intestine (DeLuca, 1967) and the formation of a calcium-binding protein in the intestinal mucosa (Wasserman & Taylor, 1966). Since its isolation, considerable evidence has accumulated suggesting that calcium-binding protein has an important role in calcium absorption (Wasserman & Taylor, 1968).

At the molecular level, however, the mechanism of action of vitamin D on the intestine is less well understood. The first evidence that protein synthesis was

essential for the action of vitamin D was provided by Eisenstein & Passavoy (1964), who showed that actinomycin D inhibited the mobilization of bone mineral in response to the vitamin. Further support for this view came from experiments showing an increased incorporation of orotic acid into RNA in response to the vitamin (Norman, 1965; Stohs *et al.*, 1967) and the report that vitamin D stimulated the DNA-template capacity of the intestinal nuclei (Hallick & DeLuca, 1969).

Some controversy still surrounds the action of actinomycin D. In rats, the antibiotic inhibits the production of 1,25-dihydroxycholecalciferol by the kidney but not its action on intestinal calcium absorption (Tanaka & DeLuca, 1971), whereas in chicks the synthesis of 1,25-dihydroxycholecalciferol is not so readily inhibited (Gray & DeLuca, 1971), but a complete inhibition of 1,25-dihydroxycholecalciferol action on intestinal calcium absorption is observed (Lawson & Emtage, 1974). This latter finding has been strengthened by the demonstration that both α -amanitin and actinomycin D inhibit the formation of calcium-binding protein by embryonic chick intestine in organ culture in response to 1,25-dihydroxycholecalciferol (Corradino, 1973a).

We investigated the mode of action of vitamin D by studying the synthesis of calcium-binding protein in a cell-free system *in vitro* and by attempting to

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correlate the findings with the hypothesis that 1,25-dihydroxycholecalciferol acts at the level of gene transcription.

The present report describes the isolation and properties of polyribosomes from chick intestinal mucosa, the preparation and specificity of the anti-serum used to detect calcium-binding protein and compares the ability of polyribosomes from deficient and vitamin D-dosed chicks to synthesize calcium-binding protein.

Experimental

Materials

Chemicals. L-[U- ^{14}C]Leucine (311 mCi/mmol) and L-[4,5- ^3H]leucine (46.9 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, GTP, creatine phosphate and creatine phosphokinase were supplied by C. F. Boehringer und Soehne, Mannheim, Germany, and heparin (sodium salt) and dithiothreitol by Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, U.K. Sucrose was the ribonuclease-free, ultra-pure variety from Schwarz-Mann, Orangeburg, N.J., U.S.A., and NCS was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bentonite was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and treated as described by Fraenkel-Conrat *et al.* (1961) before use. Purified ribonuclease inhibitor was from G. D. Searle, High Wycombe, Bucks., U.K.

Animals. Day-old chicks (Rhode Island Red $\times$ Light Sussex) were obtained from the National Institute for Research in Dairying (Shinfield, Reading, Berks., U.K.). They were fed on the cholecalciferol-deficient diet described by Lawson *et al.* (1969) and were used after 4–5 weeks. In all experiments the chicks were starved for 18h before being killed. Vitamin D-dosed chicks received 5 μg of cholecalciferol (200 i.u.) orally 72h before being killed.

Black and white hooded rats were obtained from stock and starved for 18h before being killed by decapitation.

Methods

Isolation of chick intestinal polyribosomes. Deficient or vitamin D-dosed chicks were killed by cervical dislocation, the jejunal and ileal segments of the small intestine removed and flushed with a cold 0.9% saline solution containing 100 μg of heparin/ml. All subsequent manipulations and centrifugations were at 4°C.

The mucosal tissue was scraped from the underlying muscle coat and the mucosa (7–9g wet wt. from four chicks) was homogenized either in 0.25M-sucrose containing 25mM-KCl, 5mM-MgCl₂, 50mM-Tris-HCl, pH 7.5 (TKM buffer) and 100 μg of heparin/ml or in rat liver cell-sap (5ml of medium/g of mucosa).

The homogenate was centrifuged for 10min at 20000g_{av.} in a Beckman model L centrifuge and samples (13ml) of the post-mitochondrial supernatant were layered over a discontinuous sucrose gradient containing 2.0M-sucrose (6ml) and 0.5M-sucrose (2ml). Both sucrose solutions contained the above buffer plus 150 μg of heparin/ml. The gradients were centrifuged for 4h at 160000g_{av.} in the MSE 59594 rotor with an MSE 65 ultracentrifuge. After centrifugation the supernatant was removed by aspiration, the pellets were rinsed with TKM buffer without sucrose and then resuspended in the same buffer. The polyribosome preparations had an E_{260}/E_{280} ratio of 1.80–1.85 and were stored in small portions in liquid N₂.

Preparation of rat liver cell-sap. Rat liver was homogenized in 0.25M-sucrose in TKM buffer (2.5ml/g) and the cell-sap fraction prepared as described by Falvey & Staehelin (1970). This solution was made 0.5mM with dithiothreitol and 100 $\mu\text{g}/\text{ml}$ with heparin and used as a source of RNAase (ribonuclease) inhibitor; intestinal mucosa was homogenized directly in it.

Gel-filtered cell-sap. The cell-sap fraction (10ml) from chick intestinal mucosa or rat or chick liver was passed through a column (2.5cm $\times$ 34cm) of Sephadex G-25 equilibrated in the sucrose-TKM medium. The protein front was collected and frozen in small portions.

Polypeptide synthesis in vitro. Reaction mixtures containing 1 μmol of ATP, 0.4 μmol of GTP, 7 μmol of creatine phosphate, 40 μg of creatine phosphokinase, 50nmol of each amino acid except leucine, 0.5 μCi of [^3H]leucine, 0.2ml of gel-filtered rat liver cell-sap (unless otherwise stated) and 0.2–0.5mg of polyribosomal RNA in a total volume of 1ml were incubated at 37°C for up to 30min. The final ionic concentrations were 100mM-KCl, 4mM-MgCl₂, 20mM-Tris-HCl, pH 7.5, and 1mM-dithiothreitol. Trichloroacetic acid-precipitable radioactivity was measured as described by Mans & Novelli (1961).

The percentage of radioactivity released from the ribosomes as completed polypeptide chains was determined by centrifuging the incubation mixture for 3h at 120000g_{av.} in a MSE 65 ultracentrifuge with a MSE 59592 rotor and samples of the resulting supernatant were treated as described by Mans & Novelli (1961).

Density-gradient analysis of polyribosome profiles. Chick intestinal polyribosomes were layered over a 12.5ml linear density gradient of 15–45% sucrose in TKM buffer and centrifuged for 100min at 132000g_{av.} in a Spinco L2-65B ultracentrifuge (SW 36 rotor) at 4°C. The polyribosome profiles were analysed by measuring E_{254} in a LKB Uvicord II with a 0.4cm-light-path flow cell.

When polyribosomes were fractionated after polypeptide synthesis *in vitro*, fractions (0.6ml) were

collected, 200 μ g of bovine serum albumin was added as carrier and protein precipitated with 5% (w/v) trichloroacetic acid. The precipitates were collected on Whatman GF/C glass-fibre filters, dried and counted for radioactivity in toluene-based scintillator. To measure the incorporated radioactivity at the top of the gradient, the last three fractions were pooled, incubated at 37°C for 20 min with 0.3M-NaOH and then precipitated with trichloroacetic acid.

Preparation of antiserum to calcium-binding protein. Calcium-binding protein was purified to electrophoretic homogeneity as described by Wasserman *et al.* (1968) with the modification that two DEAE-Sephadex column steps were substituted for the preparative electrophoresis. This protein was emulsified with Freund's complete adjuvant and injected subcutaneously on the backs of New Zealand White rabbits (1 mg/rabbit). They were bled from the ear vein 6–8 weeks after antigen administration and a γ -globulin fraction was prepared as described by Taylor & Wasserman (1970).

Antibody precipitation reaction. Samples obtained from polypeptide synthesis *in vitro* or from whole cell incubations (400 μ l), a solution of 10% (v/v) Triton X-100, 10% (w/v) sodium deoxycholate and 100 mM-leucine (50 μ l), purified calcium-binding protein as carrier (10 μ g) and antiserum (100 μ l; enough to precipitate 15–20 μ g of calcium-binding protein) were mixed in plastic centrifuge tubes and left at room temperature for 30 min. The precipitate was pelleted by using the Eppendorf 3200 'Zentrifuge', washed in 500 μ l of TKM buffer and re-centrifuged. The pellets were subjected to either radioactive scintillation counting or polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis. Samples (200 μ l) from polypeptide synthesis *in vitro* were separated on 7.5% (w/v) acrylamide gels at room temperature at 5 mA/gel in the Tris-EDTA-borate buffer system described by Taylor & Wasserman (1967). After electrophoresis the gels were either stained in 0.25% Coomassie Brilliant Blue or frozen in solid CO₂ and cut into 1 mm discs. The proteins in the discs were extracted by incubating with 0.5 ml of NCS at 50°C for 2½ h, then cooled and counted for radioactivity in 10 ml of toluene-based scintillator fluid.

For electrophoresis in sodium dodecyl sulphate-polyacrylamide gels, immunoprecipitates and marker proteins were dissociated by heating at 100°C for 2–3 min in 50 mM-Tris-HCl buffer, pH 6.8, 1% sodium dodecyl sulphate, 1% β -mercaptoethanol and 10% (v/v) glycerol. Gels were prepared as described by Weber & Osborn (1969), and after electrophoresis were treated as described above.

Whole cell incubations. The jejunum portion of the intestine was rinsed in ice-cold 0.9% saline, everted on a thin metal rod and sliced. The slices (10–20 mg) from deficient or vitamin D-dosed chicks were then incubated for 2 h at 37°C in basal Eagle's medium

(10 ml/g of tissue) supplemented with 10 mg of bovine serum albumin/ml and containing [¹⁴C]- or [³H]-leucine respectively. The slices were then rinsed in saline, homogenized separately in 100 mM-NaCl, 5 mM-KCl and 15 mM-Tris-HCl, pH 7.5, and centrifuged at 120 000 g_{av} for 3 h in the MSE 5952 rotor of the MSE 65 ultracentrifuge.

Measurement of radioactivity. Samples incubated with NCS and dried on filter discs were counted in 10 ml of toluene scintillator (Noll, 1969). The samples were counted in a Packard Tri-Carb Model 3375 automatic liquid-scintillation counter. Quenching was corrected by using automatic external standardization and correlation curves for ¹⁴C and ³H.

Calculations. To determine the yield of polyribosomes and the number of amino acids incorporated per ribosome during protein synthesis *in vitro* we have assumed: (a) 1 mg of polyribosomal RNA = 20 E_{260} units; (b) molecular weight of mammalian ribosome = 4×10^6 ; (c) RNA accounts for 55% of ribosomal mass.

Results

Isolation of intestinal polyribosomes

Although it is now generally accepted that polyribosomes from mammalian tissues can be isolated by sedimentation through a layer of 2.0 M-sucrose solution (Noll, 1969), the precautions required to obtain undegraded preparations from a specific tissue are not always known. Alpers & Isselbacher (1967) reported high RNAase (ribonuclease) activities in homogenates of rat intestinal mucosa and could improve both the polyribosome profiles and the protein synthetic ability of the polyribosomes either by ligation of the common bile duct or by using bentonite during the preparative procedures. In contrast, Palacios *et al.* (1972) obtained undegraded polyribosomes from chick oviduct when 100 μ g of heparin/ml was included in the homogenization medium.

From the above observations it is evident that the control of RNAase activity is an important prerequisite if good polyribosome preparations are to be obtained. Chicks were therefore starved for 18 h before use to decrease the secretion of digestive juices by the pancreas. Further, the duodenal segment of the small intestine, with the pancreas attached, was discarded and mucosa was obtained only from the jejunum and ileum. In spite of these precautions, initial attempts at isolating undegraded polyribosomes from chick intestine were unsuccessful (Fig. 1a), and resulted in polyribosome profiles typical of those obtained after RNAase treatment (Noll, 1969).

In an effort to improve the polyribosome preparations we sought to control RNAase activity by using known RNAase inhibitors. We have tried polyvinylpyrrolidone, heparin, bentonite and purified RNAase

Fig. 1. Effect of RNAase inhibitors on polyribosome preparations from chick intestine

(a) Polyribosomes isolated in the absence of any added RNAase inhibitor. (b) Polyribosomes isolated in the presence of rat liver cell-sap. For (a) and (b) the isolation procedure is described in the Experimental section and 250 μ g of polyribosomal RNA was used per gradient. The arrows indicate the position of monomers.

inhibitor from rat liver; only bentonite improved the polyribosome profiles. Unfortunately, however, the non-specific adsorptive property of bentonite resulted in low yields of polyribosomes and for this reason the use of bentonite was not continued.

The failure of heparin to improve the chick intestinal polyribosome profiles was surprising in view of the results of Palacios *et al.* (1972) and of Palmiter (1973) who used up to 1500 μ g of heparin/ml of homogenization medium to obtain intact ovalbumin mRNA from chick oviduct. Our findings are best explained by the inhibitory characteristics of heparin for different types of RNAase. Shortman (1962) has shown that heparin inhibits 'acid' RNAase activity but is without effect on 'alkaline' RNAase activity. Thus it may be postulated that the RNAase activity in the oviduct system is an 'acid' one whereas that of intestine is 'alkaline' and thus should be inhibited by the endogenous RNAase inhibitor present in rat liver.

When intestinal mucosa was homogenized in rat liver cell-sap as described in the Experimental section, the resulting polyribosome preparation (Fig. 1b) showed less sign of RNAase degradation (cf. Fig. 1a). The preparation consisted mainly of large polyribosomes with a peak at 11–12 ribosomes/polyribosome. All subsequent experiments described were conducted with polyribosomes prepared in the presence of rat liver cell-sap.

The yield of polyribosomes isolated in this fashion varied from 0.6 to 0.8 mg of polyribosomal RNA/g wet wt. of intestinal mucosa. This was not dependent on the vitamin D status of the chicks and indeed similar polyribosome profiles were obtained from both deficient and vitamin D-dosed chicks.

Amino acid-incorporating ability of intestinal polyribosomes

The kinetics of incorporation of [3 H]leucine into protein by intestinal polyribosomes is shown in Fig. 2 and the requirements for cell-free protein synthesis are illustrated in Table 1. Incorporation of amino acids, under optimum conditions, was linear for the first 8 min and then gradually declined, being complete after 30 min. Although the system exhibited an absolute requirement for Mg^{2+} (with an optimum at 4 mM- Mg^{2+}), ATP and an ATP-generating system, maximum activity also depended on GTP. Omission of the 19 unlabelled amino acids caused a significant depression in incorporation.

Since we were mainly interested in the structural integrity of the polyribosomal preparations, we investigated their ability to complete and release the nascent polypeptide chains under incubation *in vitro* and attempted to define the conditions for maximum release. In particular the effect of cell-sap from chick

Fig. 2. Kinetics of amino acid incorporation by intestinal polyribosomes

Amino acid incorporation was measured as described under 'Methods' with gel-filtered rat liver cell-sap (●) or gel-filtered chick intestinal cell-sap (○). For both experiments the concentration of polyribosomal RNA was 0.4 mg/ml.

intestine, chick liver and rat liver on the release of completed polypeptide chains by intestinal polyribosomes was determined. Only 8% of the total incorporated radioactivity was released with cell-sap from chick intestine. This low percentage release is accompanied by a rapid cessation of incorporation (Fig. 2) and substantiates our proposal that intestinal homogenates have a considerable RNAase activity. Chick liver cell-sap gave marginally better results, releasing up to 15% of the incorporated radioactivity, whereas rat liver cell-sap produced the largest release of completed chains (30–35%).

The release of completed chains with rat liver cell-sap is better illustrated in Figs. 3(a)–3(d). This shows the sucrose-gradient analysis of incorporation mixtures incubated at 37°C for various lengths of time and illustrates the change of the size distribution of polyribosomes simultaneous with the release of single 80S ribosomes and ³H-labelled protein. The release of completed chains by our intestinal polyribosome preparations, with rat liver cell-sap as a source of tRNA and activating enzymes, is slightly less than that found for mouse liver polyribosomes by Falvey & Staehelin (1970), but compares favourably with the percentage release reported by Korner (1961) and by Ragnotti *et al.* (1969) for rat liver polyribosomes.

Table 1. Requirements for protein synthesis *in vitro* by intestinal polyribosomes

The complete system is described under 'Methods'. The incubation contained 0.3 mg of polyribosomal RNA/ml and was carried out at 37°C for 30 min.

	10 ⁻⁵ × ³ H radioactivity (d.p.m./mg of polyribosomal RNA)
Complete system	12
ATP omitted	2.3
ATP-generating system omitted	1.9
ATP and ATP-generating system omitted	0.2
GTP omitted	8.4
Mg ²⁺ omitted	0
19 amino acids omitted	7.7

Specificity of anti-calcium-binding protein γ -globulin

The specificity of the antiserum was demonstrated as follows. Intestinal slices from deficient or vitamin D-dosed chicks were incubated for 2 h at 37°C in basal Eagle's medium and a supernatant fraction obtained as described under 'Methods'. Samples of each supernatant (containing ¹⁴C or ³H respectively) were mixed and an immunoprecipitate was formed by the addition of excess of antiserum. This precipitate was washed and subjected to electrophoresis on polyacrylamide gels as described under 'Methods'. Fig. 4 shows the typical distribution of radioactivity on such gels. Both the lack of any ¹⁴C in the immunoprecipitate and the single ³H radioactivity peak, with the same mobility as purified calcium-binding protein, indicate the high specificity of the precipitin reaction.

Specific protein synthesis in response to vitamin D

The ability of intestinal polyribosomes from rachitic or vitamin D-dosed chicks to synthesize calcium-binding protein in a heterologous cell-free system *in vitro* was determined by incubating polyribosomes from chick intestine or from rat liver (control) as described under 'Methods'. The ribosomes were then pelleted and the resulting supernatant was used as a source of completed polypeptide chains.

The results of this experiment, where calcium-binding protein was detected immunologically, are shown in Table 2. It is clear that intestinal polyribosomes from vitamin D-dosed chicks produce substantial amounts of a protein precipitable by the antiserum. In contrast, neither polyribosomes from rat liver, the control for non-specific absorption of radioactivity on to the immunoprecipitate, nor polyribosomes from rachitic chick intestine possessed this ability.

Definite evidence that the protein precipitated by the antiserum was indeed calcium-binding protein

Fig. 3. Kinetics of ribosome release from polyribosomes during amino acid incorporation *in vitro*

The reaction mixture was as described under 'Methods' except that $2.5 \mu\text{Ci}$ of $[^3\text{H}]\text{leucine/ml}$ and $5 \mu\text{M}$ of non-radioactive leucine were present. The polyribosome concentration was 0.4 mg of polyribosomal RNA/ml. Samples (0.5 ml) were removed after 0 min (a), $2\frac{1}{2} \text{ min}$ (b), 10 min (c) and 30 min (d) incubation at 37°C , rapidly cooled in ice and centrifuged for 100 min at $132000g_{\text{av.}}$ at 2°C through a $15\text{--}45\%$ sucrose gradient in TKM buffer. The gradients were fractionated and samples prepared for radioactivity counting as described under 'Methods'. To determine the radioactivity in the released polypeptide the top three fractions were pooled and diluted to 4 ml . Trichloroacetic acid-insoluble radioactivity in a 0.5 ml sample was determined after incubation at 37°C for 30 min with 0.3 M-NaOH . The arrows indicate the position of monomers and the hatched area represents the top three fractions; (●), ^3H radioactivity (d.p.m./fraction); —, E_{254} .

Fig. 4. Specificity of antiserum prepared against calcium-binding protein

Intestinal slices from deficient or vitamin D-dosed chicks ($5\mu\text{g}$ of cholecalciferol, 72h before death) were incubated with [^{14}C]- or [^3H]-leucine respectively and the tissues homogenized and centrifuged as described under 'Methods'. Samples of the resulting supernatants were mixed and incubated at room temperature for 30min with 1% Triton X-100 and an excess of antiserum. The resulting immunoprecipitate was washed, dissolved as described and analysed by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels. After electrophoresis the gel was frozen with solid CO_2 and 1mm slices were cut and incubated as described under 'Methods'. The arrow indicates position of the tracker dye.

Fig. 5. Molecular weight of immunoprecipitable proteins synthesized by intestinal polyribosomes

Intestinal polyribosomes from vitamin D-dosed chicks were incubated with rat liver cell-sap as described in Table 2. Immunoprecipitable material was obtained, solubilized and separated on sodium dodecyl sulphate-polyacrylamide gels as described under 'Methods'. Also shown in the figure are the electrophoretic mobilities of (a) ovalbumin (mol.wt. 43000); (b) carbonic anhydrase (mol.wt. 29000) and (c) myoglobin (mol.wt. 17300) on parallel gels.

Table 2. Effect of vitamin D on synthesis of calcium-binding protein by isolated polyribosomes

Incubations were carried out at 37°C for 30min with rat liver cell-sap and the indicated polyribosome preparations. Acid-insoluble radioactivity in the released protein corresponds to a $400\mu\text{l}$ sample. The immunoprecipitate was formed and washed as described under 'Methods'. Values in parentheses give the immunoprecipitated radioactivity as a percentage of the appropriate acid-insoluble radioactivity.

Source of polyribosomes	Acid-insoluble radioactivity in released protein (d.p.m.)	Radioactivity present in immunoprecipitation (d.p.m.)
Rat liver	56000	330 (0.6%)
Chick intestine (-vitamin D)	51200	270 (0.53%)
Chick intestine (+vitamin D)	64000	4475 (7%)

and not a higher-molecular-weight precursor was provided by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the immunoprecipitate (Fig. 5). The mobility of the radioactive peak, by comparison with those of standard proteins, corre-

sponded to a molecular weight of 27500. This agrees well with that determined by Wasserman *et al.* (1968) of 25000-28000.

Martin *et al.* (1969) reported that in addition to promoting the formation of calcium-binding protein vitamin D increases the calcium-dependent adenosine triphosphatase activity of intestinal brush-border preparations. A correlation has also been observed between the elevation of alkaline phosphatase activity and intestinal calcium absorption (Haussler *et al.*, 1970). These findings suggested that other proteins besides calcium-binding protein were synthesized in response to vitamin D. We examined this by comparing the proteins synthesized *in vitro* by polyribosomes isolated from the intestine of deficient and vitamin D-dosed chicks. Fig. 6(a) shows the radioactivity profiles of these proteins after electrophoresis on polyacrylamide gels in the Tris-EDTA-borate buffer. When the results are expressed as a ratio [^3H radioactivity (d.p.m. per slice)/ ^{14}C radioactivity (d.p.m. per slice)] it is readily seen (Fig. 6b) that only one area has an increased $^3\text{H}/^{14}\text{C}$ ratio and that this protein has an R_F value identical with that of calcium-binding protein synthesized *in vivo*. In no case have we observed other changes in this ratio.

Fig. 6. Polyacrylamide-gel electrophoresis of polypeptide chains released from polyribosomes after incubation *in vitro*

(a) Intestinal polyribosomes from deficient or vitamin D-dosed chicks were incubated with [¹⁴C]- or [³H]-leucine respectively in the cell-free system described under 'Methods'. Samples (100 μ l) of the released proteins were mixed and separated on polyacrylamide gels in the Tris-EDTA-borate buffer. Gels were sliced, incubated with NCS and assayed for radioactivity as described. ----, [³H]leucine; —, [¹⁴C]leucine. (b) ³H/¹⁴C ratios were obtained from the results of Fig. 6(a).

Discussion

In devising a method for isolating intestinal polyribosomes we tried to minimize degradation and at the same time achieve a realistic yield. Inspection of electron micrographs of intestinal epithelial cells revealed that intestinal polyribosomes existed mainly as 'free' polyribosomes and were not membrane-bound. For this reason the postmitochondrial supernatant was not detergent-treated before further centrifugation. Indeed, treatment with Triton X-100 produced only a small increase in the yield of polyribosomes and at the same time caused further degradation, presumably by rupturing the lysosomes.

The above method, coupled with the use of the endogenous RNAase inhibitor of rat liver, enables the isolation of polyribosomes from chick intestine in a relatively undegraded form (Fig. 1b) as judged by their profiles on sucrose gradients and their ability to complete and release up to 35% of the nascent polypeptide chains under optimum conditions.

The integrity of the polyribosomes may also be assessed if the number of amino acids polymerized per ribosome is known. Isotope-dilution experiments showed that the rat liver cell-sap contributes 1.5 μ M-leucine to the incubation medium (J. S. Emtage, unpublished work); thus the specific radioactivity of the [³H]leucine is 0.33 μ Ci/nmol. Fig. 2(a) indicates that maximum incorporation is 1.5×10^6 d.p.m./mg of polyribosomal RNA. Thus if it is assumed that 1 mg of polyribosomal RNA is equivalent to 450 pmol of ribosomes, it can easily be deduced that there are 4.5 leucine molecules incorporated/ribosome. Further, the assumption that 10% of newly synthesized protein is leucine and that 50% of the ribosomes in the polyribosome preparation are inactive leads to the conclusion that an average of 90 amino acids are incorporated/ribosome.

The degree of incorporation achieved with chick intestinal polyribosomes (2 nmol of leucine/mg of ribosomal RNA) is equivalent to 10 nmol of amino acid/mg of ribosomes and may be compared with the results of Jernigan *et al.* (1973), who obtained a value of 2–5 nmol of amino acid/mg of polyribosomes with chick liver polyribosomes, and of Heywood *et al.* (1968), who reported a total amino acid incorporation of 1 nmol/mg of ribosomes with embryonic chick muscle polyribosomes.

In common with most other mammalian protein-synthesizing systems that have been extensively fractionated and then reconstituted, little if any reinitiation of protein synthesis occurred with our polyribosome preparations. This failure to reinitiate results in a gradual decrease in the size of the polyribosomes accompanied by a parallel increase of single ribosomes and released polypeptide chains (Figs. 3a–3d).

In spite of this the completed chains should contain an *N*-terminal region with an average of 90 newly synthesized amino acids, corresponding to a molecular weight of approx. 9000, and ribosomes at or near the 5' end of the mRNA should polymerize peptides twice this size. Because of these relatively long stretches of RNA translated (up to 500 nucleotides), an examination of the types of protein synthesized by these ribosomes and of the effect of vitamin D on specific protein synthesis was thought to be justified.

By using the antiserum prepared against calcium-binding protein we demonstrated the formation *de novo* of that protein by intestinal polyribosomes from vitamin D-dosed chicks (Table 2). Moreover, the nature of the cell-free system, consisting of only polyribosomes from intestine, and the fact that greater than 90% of the immunoprecipitable radioactivity migrated with an apparent molecular weight of 27500 (Fig. 5) appears to rule out any direct effect of cholecalciferol (or its metabolites) on tRNA species, activating enzymes or elongation factors or

on post-transcriptional events. Synthesis *de novo* of calcium-binding protein and the inability of polyribosomes from rachitic chick intestine to produce the protein is in good agreement with the results of MacGregor *et al.* (1970), who reported direct incorporation of [³H]leucine, injected *in vivo*, into calcium-binding protein, and of Corradino (1973*a,b*), who observed bulk synthesis of calcium-binding protein by embryonic chick intestine in organ culture in response to vitamin D and its metabolites as well as an inhibition of calcium-binding protein production by both actinomycin D and α -amanitin. Our results lend further weight to the hypothesis that 1,25-dihydroxycholecalciferol is promoting the synthesis of an mRNA species that codes for calcium-binding protein.

Whether the specificity of the vitamin D effect is such that only calcium-binding-protein synthesis is induced is still open to question. It might be expected that other specific proteins, forming part of the calcium-transport mechanism, would be induced as well, and indeed there is evidence that both alkaline phosphatase and calcium-dependent adenosine triphosphatase activities of intestine are increased by vitamin D administration (Martin *et al.*, 1969; Haussler *et al.*, 1970). However, electrophoresis of the products of polyribosomal synthesis *in vitro* produced evidence for only a single specific protein, calcium-binding protein (Fig. 6). Similar results were obtained when soluble proteins produced during incubation of intestinal slices *in vitro* were separated under the same conditions (J. S. Emtage, unpublished work). Despite these negative findings, it is still impossible to state categorically that other proteins are not produced, for the following reasons: (1) membrane protein components in general are ill-defined and difficult to identify because of their insoluble nature; (2) the experiments described were conducted 72h after cholecalciferol administration. The possibility of a protein appearing before calcium-binding protein cannot be dismissed.

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