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Vitamin-A-dependent zinc-binding protein and intestinal absorption of Zn in chicks

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1. It was demonstrated that the development of experimental avitaminosis A in chicks led to secondary zinc deficiency. The balance of Zn in the chick became negative, while the Zn content of various tissues decreased. Thus in vitamin-A-deficient chicks the serum Zn content was 1258 (SD 26·3) μg/l which was considerably lower than 1652 (SD 97·8) μg/l in controls.

2. Zn absorption was considerably reduced throughout the entire small intestine of vitamin-A-deficient chicks and most markedly in the ileal region. Within 72 h after retinyl acetate administration Zn absorption was fully restored in this region of the intestine.

3. The ^{65}Zn-binding capacity of soluble proteins, present in the supernatant fraction of ileal-mucosa homogenates of vitamin-A-deficient chicks, was found to increase 2·6 times by 72 h after the administration of a single dose of retinyl acetate.

4. A vitamin-A-dependent Zn-binding protein (ZnBP), absent in vitamin-A-deficient chicks, was isolated from the ileal mucosa after their repletion with vitamin A. Competitive-binding studies (calcium, cadmium, copper) showed the protein to be highly specific for Zn ions.

5. The molecular weight of ZnBP was 83 kDa. The association constant of the protein–Zn complex was $0{\cdot}8 \times 10^6$/mol. The protein was acidic with approximately 20% of its amino acid residues belonging to dicarboxylic acids. ZnBP was found to be a glycoprotein, and it contained hexose as a carbohydrate component.

6. It is suggested that ZnBP is involved in the binding of Zn in the ileal mucosa of chicks.

Zinc belongs to a group of a biologically active trace elements, having been shown to take part in important and diverse processes in living organisms. This element is incorporated in the molecular structure of a large number of metalloenzymes: it is necessary for synthesis of nucleic acids and proteins, and for maintenance of processes involved in cell division (Hansen *et al.* 1982).

It is known that the supply of Zn to humans or animals does not depend only on its concentration in food. Multiple nutritional factors appear to be essential in defining its availability from various diets. Some amino acids, phytic acid and a surplus of calcium, phosphorus, iron, and other metals have been shown to reduce the uptake of Zn, leading to the development of a secondary deficiency of Zn (House *et al.* 1982; Solomons, 1982; Forbes *et al.* 1984). On the other hand, histidine, cysteine and, to a somewhat lesser extent, threonine (Dahmer, 1971; Schwarz & Kirchgessner, 1975) and tryptophan (Evans, 1980), stimulate the absorption of Zn from foods.

Our previous investigations (Valinietse *et al.* 1975; Bauman & Berzin, 1976) showed that reduced Zn absorption in the small intestine was the principal disturbance in Zn metabolism of vitamin-A-deficient chicks. A detailed examination of the effect of vitamin A on the absorption of Zn in chick small intestine was the aim of the present study.

EXPERIMENTAL DESIGN AND METHODS

All animals used in these experiments were White Leghorn cockerels. From the beginning of the experiment chicks were fed on a basal diet of the following composition (g/kg): barley meal 510, oatmeal 210, peanut oilcake 170, casein 50, yeast 30, bone flour 15, chalk 10, common salt 5, vitamin D_3 in oil 12·5 μg/kg. The diet contained no vitamin A, the digestible protein was 171·0 g/kg and the Zn content 23·0 mg/kg.

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The chicks were divided into three main groups. The control group was given *ad lib.* the diet containing retinyl acetate (2752 μg/kg) added in oil to the basal diet in accordance with the recommended dosage (Bruckental *et al.* 1974). The second group received the vitamin-A-free basal diet (−A chicks). The third group consisted of vitamin-A-deficient chicks that were given orally 3440 μg retinyl acetate in oil 24–72 h before death (+A chicks). Some of the +A chicks were pair-fed with −A chicks, and each member of the pair-fed subgroup received 16 g food/d, the amount equivalent to that consumed by an individual −A chick.

The animals were given the experimental diets for 4·5–5 weeks. By then the growth of −A chicks had sharply decreased and they had started to show signs of avitaminosis A. During the course of the experiment, chick growth and the extent of avitaminosis were regularly checked. The vitamin A content of the liver and blood serum levels of uric acid served as criteria to judge the degree of avitaminosis A in chicks (Bruckental *et al.* 1974). Retinol was determined by spectrophotometry according to Dimitrovsky & Kryukov (1975), and uric acid by a modification of the method of Foline & Denis (Eichhorn *et al.* 1961).

Metabolic balance studies

In order to study the action of vitamin A on Zn absorption and metabolism, Zn balance was determined daily for the last 3 d of the experiment. Food and water consumption was monitored, and faeces were collected quantitatively (group method, twenty chicks/group). Samples of food, water and faeces were then analysed to determine their Zn contents. To calculate Zn balance or its absorption, the amount of the element in the faeces was subtracted from Zn in the food and water consumed. All quantitites were expressed per kg body-weight per d.

Zn absorption in the small intestine

Zn absorption was studied *in situ* in all three regions of the small intestine: duodenum, jejunum and ileum. Control, −A and +A chicks (ten chicks/group) were used in the experiment. Before the absorption studies, chicks were fasted for 16–18 h. During the experiment, chicks were kept under diethyl ether anaesthesia. An isolated intestinal loop of ligated gut sac, 50 mm long, was filled with 0.5 ml buffer solution. Buffer composition (mM) for duodenal and jejunal sacs was: 4 Tris hydrochloride, 6 zinc chloride, 145 sodium chloride, 4 potassium chloride, 20 fructose, pH 7·4; in buffer solutions infused into ileal sacs $ZnCl_2$ was replaced by ^{65}Zn (0·1 μCi). After 1 h chicks were decapitated and isolated segments removed. The contents were drained into a test-tube, segments were rinsed with 1 ml 154 mM-NaCl and put into a separate test-tube. The amount of Zn absorbed was calculated as the difference between Zn content just after the infusion and 60 min later, and expressed as nmol/g tissue. The percentage of ^{65}Zn absorbed was calculated according to the following expression:

$$\text{absorbed } ^{65}\text{Zn } (\%) = \frac{^{65}\text{Zn infused} - {}^{65}\text{Zn non-absorbed}}{^{65}\text{Zn infused}} \times 100$$

Zn binding in mucosa of the small intestine

Zn binding by the intestinal wall was studied by means of mucosal-accumulating preparations as developed by Ugolev *et al.* (1970) for investigation of the first stages in transport processes. An everted intestinal segment, mounted on a glass rod, was submerged in 5 ml oxygenated Tris-buffer containing 60 μM-Zn and incubated for 10 min at 37°. The amount of accumulated Zn was calculated as the difference between the Zn contents of the mucosa before and after incubation.

Zn binding by the supernatant fractions of mucosal homogenates was determined in two

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ways. First, Zn binding was assessed by use of Chelex-100 ion-exchange resin (Bio Rad, USA) and a radioisotopic technique as described by Wasserman *et al.* (1968) for determination of Ca-binding activity in the intestinal epithelium. The procedure depends on the competition between an ion-exchange resin and supernatant fractions of homogenates for added ^{65}Zn. Mucosal homogenates (200 mg/ml) were prepared in Tris-buffer (pH 7·4) and centrifuged at 100000 *g* for 1 h in a refrigerated centrifuge VAC-601 (Janetzky, GDR). Then, 0·5 ml supernatant fraction was mixed with 1 ml buffer, 0·2 ml Chelex-100 suspension (150 g/l), and 0·05 ml $^{65}ZnCl_2$ (1 μCi). Samples were shaken and 5 min later centrifuged at 600 *g* for 10 min; ^{65}Zn in the supernatant fraction was subsequently assayed. Protein was determined by its absorbance at 280 nm and relating this to a standard curve prepared for human serum albumin. In the absence of soluble proteins from the mucosa, practically all the ^{65}Zn was sequestered by the resin and was detectable in the pellet. When portions of the supernatant fractions of mucosal homogenates were added some part of the ^{65}Zn was bound by the soluble proteins present in that fraction. The Zn-binding activity (ZnBA) of the proteins was calculated as the percentage of ^{65}Zn complexed with 1 mg proteins.

The second method for measuring Zn-binding was the flow dialysis method proposed by Colowick & Womack (1969) and slightly modified for use with ^{65}Zn. The flow dialysis cell was made of Teflon and consisted of two sections, the upper and lower, separated by a semi-permeable cellophane membrane. Samples (3 ml) to be assayed (supernatant fractions or purified protein solutions) were placed in the upper section followed by 1·6 μCi ^{65}Zn as the chloride. The buffer solution (14 mM-Tris hydrochloride, 119 mM-NaCl, 4·7 mM-KCl, pH 7·4) was pumped through the lower section at a constant rate (4 ml/min). The mixture of ^{65}Zn with portions of supernatant fractions or protein solutions was titrated with increasing amounts of stable Zn and its capacity to replace ^{65}Zn, complexed with protein, was determined according to the level of radioactivity in the dialysate. Precision pipettes (Hamilton) were used to place 10–50 μl volumes of Zn solutions in the flow dialysis cell. Fractions (20 s) of the dialysate were collected, and the radioactivity level was subsequently determined in each one.

Effect of vitamin A on Zn-binding activity of intestinal soluble proteins

Action of retinyl acetate on ^{65}Zn binding by the mucosal soluble proteins was studied in three regions of the intestine: duodenum, jejunum and ileum. Two groups of chicks were used in the experiment: −A chicks, and +A chicks repleted with retinyl acetate 72 h before death. Each group consisted of fifteen birds. After decapitation the small intestine was removed, the mucosa scraped off the three regions and homogenates prepared to obtain the soluble-protein fraction by the previously described method. The total protein was determined by the Biuret reaction (Gornall *et al.* 1949). Ion-exchange Chelex-100 resin and radioactive isotopes were used to measure ZnBA of proteins as described earlier. To examine the effect of retinyl acetate dosage on ^{65}Zn binding by soluble proteins in the ileal mucosa, chicks were divided into eight groups of ten to twelve birds each. The first group served as vitamin-A-deficient controls and did not receive the vitamin at all. Seven other groups were given orally 28, 76, 138, 344, 1376, 3440, and 6880 μg retinyl acetate in oil 72 h before death. Ion-exchange Chelex-100 resin and radioactive isotopes were used to determine ZnBA as described earlier.

Time-dependence of ^{65}Zn binding by soluble proteins in the ileal mucosa after retinyl acetate administration (3440 μg) was studied in five groups of chicks (ten birds each): (1) −A chicks, (2) −A chicks, repleted with retinyl acetate 24 h before death, (3) 48 h before death, (4) 72 h before death, (5) 166 h before death. The soluble protein fraction was obtained and ZnBA determined as stated previously.

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Purification of Zn-binding protein from the chick ileal mucosa

To isolate the Zn-binding agent the supernatant fraction obtained from 4 g mucosa (approximately 16 ml) was desalted on a Sephadex G-25 column, the resulting fluid lyophilized, the dry protein obtained redissolved to achieve a concentration of 50 g/l, and then subjected to gel-filtration column chromatography on Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden), employing a 16 × 500 mm glass column; Sephadex gel was previously equilibrated in 14 mM-Tris hydrochloride buffer, pH 7·4. Ionic Zn, as 10^{-4} M–$ZnCl_2$ or ^{65}Zn (0·2 μCi/mg total protein), was added to the supernatant fraction 1 h before gel filtration. The column was run with equilibration buffer by means of a peristaltic pump; the flow-rate was 26 ml/h. Fractions (8 min) were collected and protein content determined spectrophotometrically by a single-beam detector at 254 nm. Zn or ^{65}Zn were also measured.

To examine the specificity of Zn binding by partially purified Zn-binding protein (ZnBP), the capacity of various bivalent cations (copper, cadmium, and Ca) to displace Zn, in a competitive-binding situation, from the protein was assessed in the flow-dialysis cell. All investigated cations were added to the cell as chloride salts.

Samples of soluble proteins (50 mg protein) from the ileal mucosa previously incubated for 1 h with Cu^{2+} as 10^{-5} M-$CuSO_4$ and Cd^{2+} as 5×10^{-5} M-cadmium chloride, were subjected to gel filtration on a column (16 × 500 mm) packed with Sephadex G-100. Protein and cation contents were determined in each fraction collected.

Sephadex G-100 columns were calibrated with standard marker proteins of known molecular weight (kDa): rabbit muscle aldolase (*EC* 4.1.2.13; 160), bovine serum albumin (67), ovalbumin (45), chymotrypsinogen (25), cytochrome (12·3) (Serva, FRG).

Final purification of ZnBP was achieved by ion-exchange chromatography (Ultrograd System, LKB), employing columns (16 × 250 mm) packed with TEAE-23 cellulose chloride; pore size 20–300 μm (Serva, FRG). Before use the columns were equilibrated with 14 mM-Tris hydrochloride buffer, pH 7·0 (conductance 0·8 mho). Each column was loaded with 50–90 mg protein from a high-molecular-weight fraction obtained by gel filtration. The retained proteins were eluted with a linear gradient produced by mixing two solutions: 14 mM-Tris hydrochloride, pH 7·0, and 14 mM-Tris hydrochloride, pH 4·5, plus 0·2 M-NaCl. The second eluent was 14 mM-Tris hydrochloride, pH 4·5, plus 1 M-NaCl, and it was used to achieve a release of strongly bound proteins. The linear flow-rate used was 42 ml/h. The eluted fractions were assayed for u.v. absorption at 280 and 206 nm. Fractions containing proteins were lyophilized, desalted on a Sephadex G-25 column, analysed by polyacrylamide gel electrophoresis (PAGE; 75 g acrylamide/l) and assessed for ^{65}Zn binding by means of the flow-dialysis method.

The protein composition of the fraction obtained by gel filtration or ion-exchange chromatography was studied by PAGE (75 g acrylamide/l). To prepare gels, 0·375 M-Tris hydrochloride buffer (pH 8·9) was used. The composition of the electrode buffer mixture was 5 mM-Tris hydrochloride, 38 mM-glycine, pH 8·3. Electrophoresis was run in 100 × 6 mm tubes (voltage 450 V, current 5 mA/tube) for 1·5 h. Each tube was loaded with approximately 100 μg protein in 100 g sucrose/l. Protein bands in gels were visualized with Coomassie G-250 (Holbrook & Leaver, 1976). Counter-ion electrophoresis was used ($^{65}Zn^{2+}$ as a counter ion) to identify ZnBP in electrophoretograms (Ueng & Bronner, 1979). In this procedure labelled Zn ($^{65}ZnCl_2$) was added to the lower (anode) chamber reservoir, and the protein sample was applied to the gel in the upper (cathode) chamber reservoir. As the ^{65}Zn migrated toward the cathode and the proteins moved toward the anode, Zn-binding sites in the gel became labelled with ^{65}Zn and could be identifed with a γ-scintillation counter. Proteins in gels were stained, gel discs of equal length prepared, and their γ-activity levels determined.

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Physico-chemical properties of ZnBP

The molecular weight of ZnBP was determined electrophoretically in the presence of sodium dodecyl sulphate (King & Laemli, 1971). Marker proteins were used of known molecular weight (kDa): phosphorylase *b* (*EC* 2.4.1.1; 94), bovine serum albumin (67), ovalbumin (45), carbonic anhydrase (EC 4.2.1.1; 30), trypsin inhibitor (201) and α-lactalbumin (14.4) (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

To determine Zn-binding indices of the protein the flow-dialysis method was applied. The estimate of the association constant (k_a) was calculated using Scatchard-plot methodology (Colowick & Womack, 1969).

Analysis of amino acid composition was performed on an automatic analyzer AAA 881 (Experimental plant of the Czechoslovakian Academy of Science). ZnBP was hydrolysed under nitrogen in 6 M-hydrochloric acid for 18 h at 105° (Spackman *et al.* 1958). The content of cysteic acid in samples was determined after the oxidation of the protein by performic acid. The carbohydrate content in the protein molecule was determined by a colorimetric method (Dubois *et al.* 1956), based on the phenol–sulphuric acid reaction with sugars and related substances. The colours produced by the reaction are stable and possess a definite absorption peak. The pentose sugars, their methylated derivatives, and their methyl-glycosides show selective absorption at 415 nm. The pentoses, methylpentoses, and uronic acids have an absorption maximum at 480 nm, while hexoses have their absorption maximum at 490 nm. The amount of colour produced at a constant phenol concentration is proportional to the amount of sugar present. Glucose was used as a standard to determine the carbohydrate content of ZnBP.

Element assays

In a number of organs and tissues, intestinal preparations, blood serum, pancreas and wing feathers, the Zn contents were determined. Blood serum proteins were denatured with trichloroacetic acid (120 g/l) and removed by centrifugation for 10 min at 600 ***g***. Samples of food, faeces, organs and tissues were dried at 105° to constant weight, ashed in a muffle furnace at 450°, and subsequently dissolved in 6 M-HCl. Zn was determined using an atomic absorption spectrophotometer (model no. 403; Perkin-Elmer). The standard solutions of Zn and samples for performing the analysis were prepared according to the instruction manual (Perkin-Elmer Corporation, 1976). The contents of Ca, Fe, Cu and Cd in the solutions were also determined with the atomic absorption spectrophotometer. In experiments with ^{65}Zn, γ-activity measurements of samples and standards were carried out in a universal well-type γ-crystal-scintillation counter USD-1 with a sodium iodide crystal. The results were assessed statistically by *t* tests. Means and standard deviations and significance values were calculated.

RESULTS

Development of avitaminosis A and Zn deficiency in chicks

Values for body-weight and some other biochemical indices (Table 1) reflected the intake of vitamin A and Zn. At the age of 5 weeks, −A chicks, raised on a diet without vitamin A, manifested signs of severe avitaminosis A: their body-weight was about 60% of that of the controls and retinol was not detected in the liver. There was a considerable increase in serum uric acid in −A chicks, characteristic of avitaminosis A in birds (Bruckental *et al.* 1974). The results also showed that −A chicks became Zn deficient. The Zn contents of serum, the pancreas and feathers were decreased by 24, 12·5 and 46% respectively. In contrast, blood serum levels of other bivalent elements (Ca, Cu, Cd, Fe) did not change

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Table 1. *The effect of vitamin A on body-weight, the serum content of uric acid and zinc metabolism in 5-week-old chicks receiving control or vitamin-A-free (−A) diets*

(Mean values and standard deviations; no. of chicks/treatment in parentheses)

	Body-wt (g)			Zn content in blood serum (mg/l)			Serum uric acid (mg/l)			Zn content in tissues						Zn balance (mg Zn retained/ kg body-wt per d)		
										Pancreas (mg/g dry wt)			Wing feathers (mg/g dry wt)					
Group	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*
−A chicks	146·6***	8·8	(20)	1·26***	0·026	(12)	120·0***	18·8	(10)	0·21*	0·01	(12)	0·081***	0·006	(12)	−0·39***	0·20	(20)
Control	250·2	3·6	(20)	1·65	0·098	(12)	25·7	2·0	(10)	0·24	0·008	(12)	0·149	0·010	(12)	1·12	0·15	(20)

Mean values were significantly different from control values: * $P < 0·05$, *** $P < 0·001$

Table 2. *Effect of vitamin A on the content of bivalent cations (μg/l) in the serum of chicks receiving control or vitamin-A-free (−A) diets*

(Mean values and standard deviations for ten birds/group)

	Copper		Cadmium		Iron		Calcium (mg/l)	
Group	Mean	SD	Mean	SD	Mean	SD	Mean	SD
−A chicks	292·0	13·5	15·1	0·7	4680	220	10·36	0·14
Control	280·0	16·0	15·0	1·1	4660	120	10·60	0·09

Values for −A chicks were not significantly different from control values.

Table 3. *The effect of vitamin A on the absorption of zinc from intestinal loops* in situ *and on the accumulation of Zn by mucosa of inverted intestinal segments in vitro (nmol/g) for chicks receiving control or vitamin-A-free (−A) diets and −A chicks receiving orally 3440 μg retinyl acetate (+A)*

(Mean values and standard deviations for ten observations/group)

	Control		−A chicks				+A chicks			
	Absorption		Absorption		Accumulation		Absorption		Accumulation	
Region of the small intestine	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Duodenum	27·5*	3·1	20·4	1·2	25·6	1·4	26·3^{NS}	5·1	30·2*	1·3
Jejunum	35·2*	5·4	21·8	1·8	32·1	2·1	32·2^{NS}	7·0	33·4^{NS}	1·3
Ileum	57·6***	7·5	24·9	4·9	33·6	3·3	69·7***	7·2	55·9 ***	2·9

NS, not significant.
Mean values were significantly different from those for −A chicks: * $P < 0·05$, *** $P < 0·001$.

significantly (Table 2). Results of balance studies demonstrated that in −A chicks the faecal output of Zn was higher than the Zn intake in food, i.e. Zn balance in their case was negative.

Effect of vitamin A on the absorption and accumulation of Zn in the chick small intestine

In situ studies on Zn absorption in the various regions of the small intestine (duodenum, jejunum and ileum) showed that in the case of −A chicks, Zn absorption was depressed throughout the entire small intestine (Table 3). A particularly high difference was observed in the distal region, the ileum, notable for intensive Zn uptake in chicks of the control group. Duodenal Zn absorption in −A chicks was reduced by 26% compared with the controls ($P < 0·05$), whereas the decrease in ileal Zn absorption was 57% ($P < 0·001$). A stimulating effect of vitamin A on Zn absorption manifested itself in −A chicks repleted orally with a single dose of retinyl acetate (+A chicks). At 72 h after the administration of vitamin A, ileal Zn absorption increased 2·8 times ($P < 0·001$) and was slightly higher than that in the control group. In the two other regions of the small intestine of +A chicks (duodenum and jejunum), the effect of vitamin A was not so evident; the increase of Zn absorption was not significant when compared with the absorption in the corresponding region of the small intestine of −A chicks.

Similar results were obtained when studying Zn accumulation by everted intestinal

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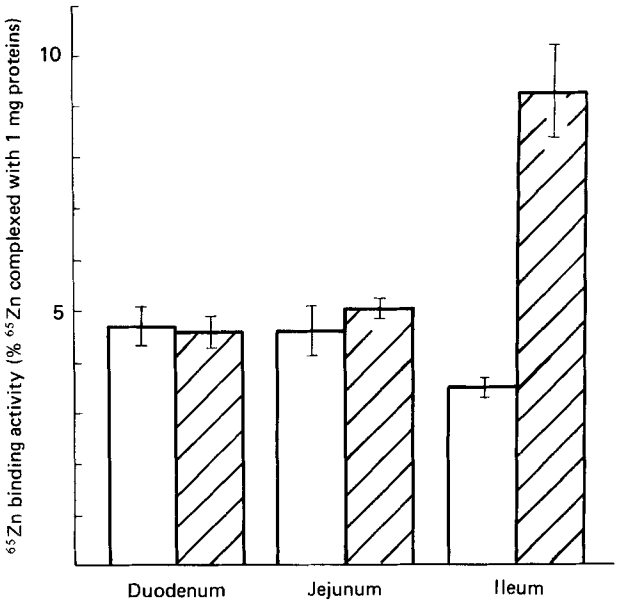

Fig. 1. Influence of vitamin A on zinc-binding activity of soluble proteins from various regions of the small intestine of chicks receiving a vitamin-A-free diet (−A) and −A chicks receiving orally 3440 μg retinyl acetate in oil (+A). (□), −A chicks; (▨), +A chicks. All values are means with their standard errors represented by vertical bars.

segments. The results in Table 3 show that after the administration of retinyl acetate to vitamin-A-deficient chicks, ileal Zn accumulation considerably increased and exceeded that in the duodenum and jejunum by 1·9 ($P < 0·001$) and 1·7 ($P < 0·001$) times respectively. In contrast the Zn absorption by ileal mucosa of vitamin-A-deficient chicks did not differ significantly from that in the two other regions of the small intestine and remained on a low level ($P < 0·5$). The accumulation of Zn in this region was slightly increased in comparison with the duodenum.

The binding of ^{65}Zn by soluble proteins from various regions of chick small intestine

To obtain additional information about ZnBA of supernatant fractions of intestinal mucosa, homogenates and added ^{65}Zn were fractionated on the ion-exchange resin Chelex-100. In the absence of soluble proteins from intestinal mucosa, Chelex-100 resin sequestered 100% of ^{65}Zn dose, and all ^{65}Zn was detectable in the pellet. In the case of high ZnBA the proteins of the supernatant fraction successfully competed with Chelex-100 resin for added ^{65}Zn, and some part of ^{65}Zn remained dissolved in solutions. Results of these investigations (Fig. 1) showed that the administration of 3440 μg retinyl acetate to −A chicks resulted, 72 h later, in a considerable increase ($P < 0·001$), 2·6 times, in ZnBA of soluble proteins from the supernatant fraction of ileal homogenates, whereas in other regions of the intestine there were no significant changes. These results demonstrate that the effect of retinyl acetate on absorption and binding of Zn by intestinal mucosa was localized in the distal region of the small intestine.

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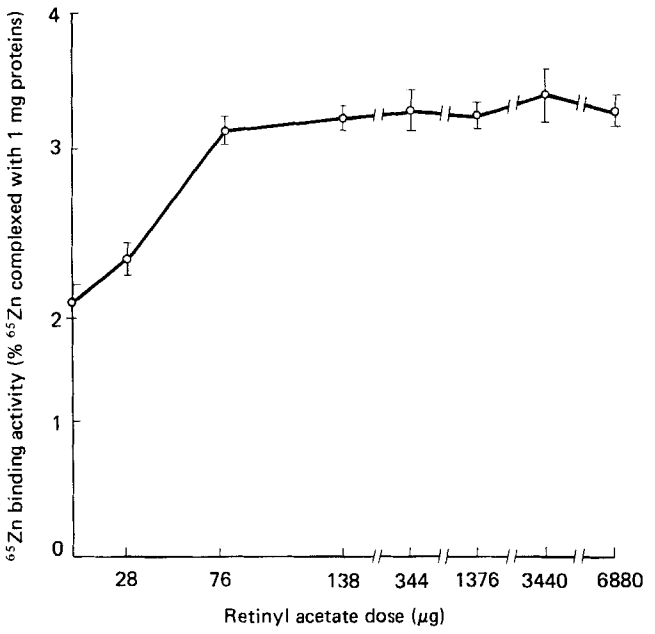

Fig. 2. Dependence of zinc-binding activity of soluble proteins from the supernatant fraction of chick ileal homogenates on the dose of retinyl acetate. Points are mean values with their standard errors represented by vertical bars.

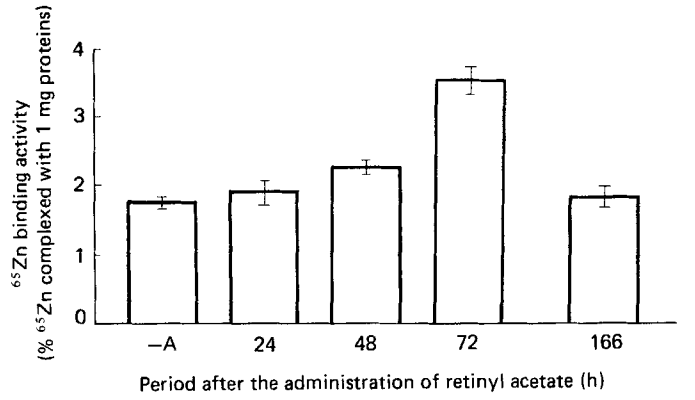

Fig. 3. Time-course of zinc-binding activity of soluble proteins from the chick ileum before (vitamin A deficient, −A) and following the administration of retinyl acetate (3440 μg). All values are means with their standard errors represented by vertical bars.

Dose and time-dependence of vitamin A action on Zn binding by the soluble proteins of the ileal mucosa

There was an increase in Zn binding by soluble proteins from the ileal mucosa with vitamin A doses up to 76 μg (Fig. 2). With doses ranging from 76 to 6880 μg the binding activity remained constant.

At 48 h, after the administration of a single dose of retinyl acetate (3440 μg) to −A chicks the Zn-binding capacity was increased ($P < 0{\cdot}05$). However, a maximum was achieved 72 h after administration (Fig. 3).

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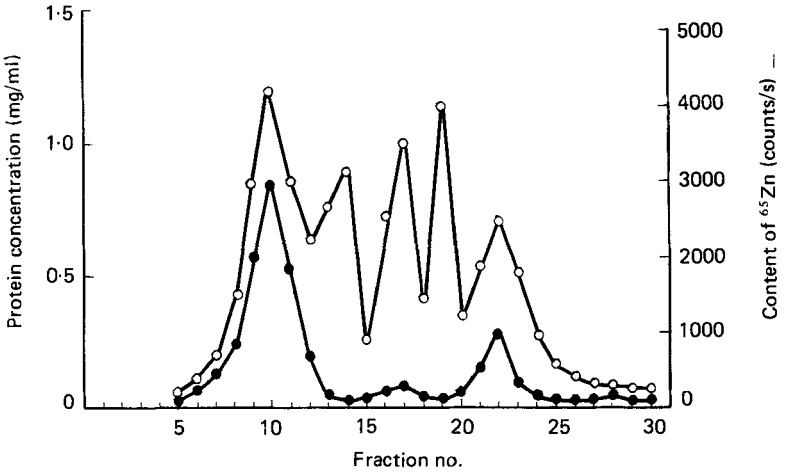

Fig. 4. Distribution of soluble proteins (○) and ^{65}Zn (●) in fractions obtained by gel filtration on Sephadex G-100 of supernatant fractions from ileal homogenates of vitamin-A-deficient chicks receiving orally 3440 μg retinyl acetate. The column was 16 × 500 mm, flow-rate 0·44 ml/min, eluant 0·014 M-Tris hydrochloride buffer, pH 7·4.

Isolation of ZnBP from ileal mucosa of chicks

In order to isolate and identify the agent responsible for the binding of Zn in the intestinal epithelium a variety of methods from the field of protein chemistry (gel filtration, ion-exchange chromatography and electrophoresis) were used.

Fractionation by gel filtration of supernatant fractions from +A chicks revealed in one of the eluted fractions (fraction 10) a protein peak containing a considerable amount of Zn (Fig. 4). Measurements of Zn-binding to proteins of this fraction by flow dialysis showed that the dialysis rate of ^{65}Zn added to the fraction from +A chicks was significantly lower than in the same fraction from −A chicks. Accordingly, ZnBA in +A chicks was 1·9 times higher ($P < 0·001$). Adding Cd, Cu or Ca in amounts necessary to reach concentrations equal to or twice that of Zn did not significantly change the dialysis rate of ^{65}Zn. It confirmed the specificity of Zn binding by the proteins from this fraction.

Gel filtration of supernatant fractions, previously incubated with Cu or Cd, on columns packed with Sephadex G-100, revealed that these cations were eluted together with proteins having molecular weights ranging from 8 to 12 kDa, whereas Zn was found to be complexed with a protein of a much higher molecular weight. Gel-filtration studies indicate it to be approximately 80-90 kDa.

PAGE (75 g acrylamide/l) of fraction 10 from +A chicks revealed in the upper half of the gel, in addition to the three protein bands, the presence of a protein which was absent in fractions from −A chicks (Plate 1). After counter-ion electrophresis, the ^{65}Zn content was determined using acrylamide gels. The additional protein band, detected in gels from +A chicks (Plate 1(*b*)), was found to bind 3·0 times more ^{65}Zn than the same region in gels from −A chicks ($P < 0·01$) (Plate 1(*a*)) and approximately 1·7 times more than the two closest accompanying protein bands. These results and those obtained previously contributed to the identification of the newly detected additional protein as a soluble, vitamin-A-dependent specific ZnBP.

Fraction 10 was separated into six protein peaks by ion-exchange chromatography (Fig. 5). ZnBP was eluted from the column in the third peak at 0·1 m-NaCl. The protein purity

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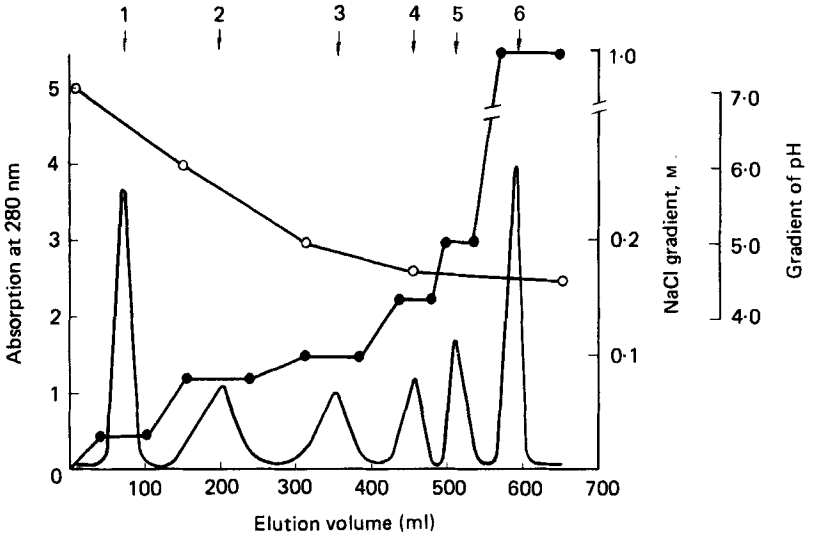

Fig. 5. Elution profile of fraction no. 10, obtained by gel filtration on a strongly basic ion-exchange resin. Column 16 × 250 mm, ion-exchange resin TEAE-23 cellulose (ionic form, chloride; capacity 0·7 meq/dry g; Serva, FRG); starting buffer 14 mM-Tris hydrochloride buffer, pH 7·0. The elution gradient was produced by means of a gradient mixer Ultrograd 11300 (LKB, Sweden), eluant 14 mM-Tris hydrochloride buffer. (○), Decreasing gradient of pH from 7·0 to 4·5; (●), increasing ionic concentration gradient from 0 to 0·2 M-sodium chloride. Linear flow-rate 42 ml/h. Fractions of 9·5 ml were collected. Protein was determined spectrophotometrically at 280 nm (——). Absorption units are arbitrary.

Table 4. *Amino acid composition of zinc-binding protein isolated from chick intestinal mucosa*

	No. of residues/ protein molecule
Amino acid*	
Lysine	27
Histidine	10
Arginine	14
Aspartic	56
Threonine	37
Serine	62
Glutamic	69
Proline	40
Glycine	115
Alanine	83
½-Cystine	10
Valine	62
Methionine	0
Isoleucine	33
Leucine	56
Tyrosine	Traces
Phenylalanine	18
Hydrophobic residues (mmol/mol)	420
No. of positively charged residues	51
No. of negatively charged residues	125

* Tryptophan was not determined.

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was established by electrophoresis. The high ZnBA (k_a 0·8 × 10^6/mol) served as additional evidence that the sample contained only ZnBP.

Effect of decreased food intake by +A chicks on ZnBP content in ileal epithelium

At 3 d after the beginning of pair-feeding, the body-weight of +A chicks which were allowed to consume the same amount of food as −A chicks was slightly higher than the body-weight of −A chicks (160 (SD 11) g for pair-fed +A chicks and 147 (SD 9) g for −A chicks). In pair-fed +A chicks the serum Zn content was 1620 (SD 59) μg/l compared with 1650 (SD 98) μg/l in chicks of the control group which were fed *ad lib.* ZnBP, absent in intestinal epithelium of −A chicks, was found to be present in pair-fed +A chicks. The presence of the protein in ileal mucosa was established using methods described in the previous section.

Some physico-chemical properties of the vitamin-A-dependent ZnBP

The molecular weight of ZnBP was defined more precisely by means of PAGE (10 g acrylamide/l) and was found to be equal to 83 kDa. Electrophoresis of the protein sample treated with sodium dodecyl sulphate in the presence of dithiothreitol (50 g/l) revealed that a molecule of ZnBP consisted of a single polypeptide chain. Comparing the electrophoretograms of the purified ZnBP (Plate 1(*c*)) and the fraction obtained after gel filtration (Plate 1(*b*)) revealed no significant changes in the position of the protein bands.

Results of the amino acid analysis of ZnBP are shown in Table 4. The protein contains a high proportion of dicarboxylic acids (aspartic and glutamic acids) and a relatively high content of leucine, alanine and glycine. There are few aromatic and sulphur-containing amino acids in the structure of ZnBP. According to the results of the amino acid analysis the molecular weight of the ZnBP is approximately 80 kDa.

Purified ZnBP was assayed for the presence of carbohydrates and it was found that the Zn-binding agent was a glycoprotein. The colorimetric method used showed that the absorption curve was characteristic of hexoses. It appeared that 1 mol protein contained two mol hexose (approximately 0·5% of the protein weight).

DISCUSSION

We can conclude that vitamin A is necessary for the absorption of Zn in chicks. The development of avitaminosis A in birds is accompanied by secondary Zn deficiency; Zn balance becomes negative and its levels in tissues decrease.

The primary site of action of vitamin A on Zn metabolism is obviously its absorption in the small intestine. In experiments using *in situ* and in vitro techniques, we have convincingly shown that in vitamin-A-deficient chicks the ileal absorption of Zn is sharply depressed. However, it is restored to normal 72 h after the administration of a single dose of retinyl acetate.

It is to be noted that in spite of intensive studies our understanding of Zn absorption in the animal intestine is still incomplete, and experimental information often contradictory. A series of in vitro experiments (Kowarski *et al.* 1974) showed the transport of Zn across the intestinal wall to be an active process requiring the presence of oxygen and metabolic sugar. It is inhibited by the substitution of sodium with lithium, by N_2 and metabolic poisons. However, not all results tend to support the presence of active Zn transport in the intestinal wall. Thus, Sahagian *et al.* (1967) did not find any effect of metabolic poisons on Zn absorption and, in their experiments, the process did not take place against an electrochemical gradient. Recent studies on the transport of Zn with isolated brush-border membrane vesicles from rats showed that ATP did not stimulate transport in this system.

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Authors of that work suggested that Zn crossed the brush-border membrane by a passive diffusion route (Menard & Cousins, 1983). On the other hand the results of Zhigure & Babarikin (1975) as well as our own results (Berzin & Bauman, 1982) demonstrate the presence of the active transport of Zn in the intestinal wall. The translocation of the cation proceeds in two stages: the first is the accumulation of the element in mucosa, and the second is its translocation into the serosal media (Sahagian *et al.* 1967). Our earlier studies of ^{65}Zn transport (Berzin, 1981) showed that retinyl acetate stimulated the translocation of ^{65}Zn into serosal media. Thus, summing up the previous results and those obtained in the present study, our investigations showed that retinyl acetate stimulated the absorption of Zn at both stages of the process. The reasons for the effect may be the presence of vitamin-A-dependent proteins or other related substances in ileal mucosa. However, at the present state of knowledge it cannot be excluded that vitamin A stimulates the absorption of Zn by some other process not involving Zn-binding factors in the intestinal mucosa.

The facts that (*a*) retinyl acetate significantly enhances both the accumulation and transport of Zn in a definite region of the small intestine, (*b*) the effect of retinyl ester develops over a period of days, (*c*) the repletion of chicks with retinyl acetate leads to the appearance of ZnBP in the ileal mucosa a few days later, and (*d*) the presence of the protein does not depend on changes of food intake after repletion, suggest that the new ZnBP depends on the presence of vitamin A in chicks. The protein binds Zn ions with high specificity (k_a $0{\cdot}8 \times 10^6$/mol). Cations like Cd^{2+}, Cu^{2+} and Ca^{2+} cannot compete with Zn^{2+} for binding sites on ZnBP. The amino acid composition of ZnBP is characteristic for glycoproteins of epithelial origin. It contains a high proportion of acidic and hydrophobic amino acid residues. At the same time there are only few aromatic and S-containing residues in the protein molecule. We found that ZnBP had a carbohydrate component.

According to previous authors (Van Campen & Kowalski, 1971; Kowarski *et al.* 1974) there are several ZnBP of different molecular weights in the small intestine of animals. Some authors suggest that the absorption of Zn is mediated by metallothioneins (Richards & Cousins, 1977; Starcher *et al.* 1980; Jackson *et al.* 1981) or by substances of low molecular weight such as prostaglandin E_2 (Evans & Johnson, 1977), citrate (Lönnerdal *et al.* 1980) or picolinic acid (Evans, 1980; Evans & Johnson, 1981). However, the presence of a vitamin-A-dependent ZnBP in the intestine with a molecular weight of 83 kDa has been shown for the first time in the present study. Further work will be necessary to clarify the character of the protein and its role in the absorption of Zn. However, it could be supposed that ZnBP enhances the binding of Zn in the ileal mucosa of chicks.

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EXPLANATION OF PLATE

Electrophoretograms of proteins from supernatant fractions of mucosal homogenates from the ileum following gel filtration for (*a*) vitamin-A-deficient chicks (−A chicks) and (*b*) vitamin-A-deficient chicks receiving orally 3440 μg retinyl acetate (+A chicks). (*c*) Isolated zinc-binding protein (**ZnBP**). Electrophoresis was performed using polyacrylamide gel (75 g acrylamide/l) with glycine buffer, pH 8·3.

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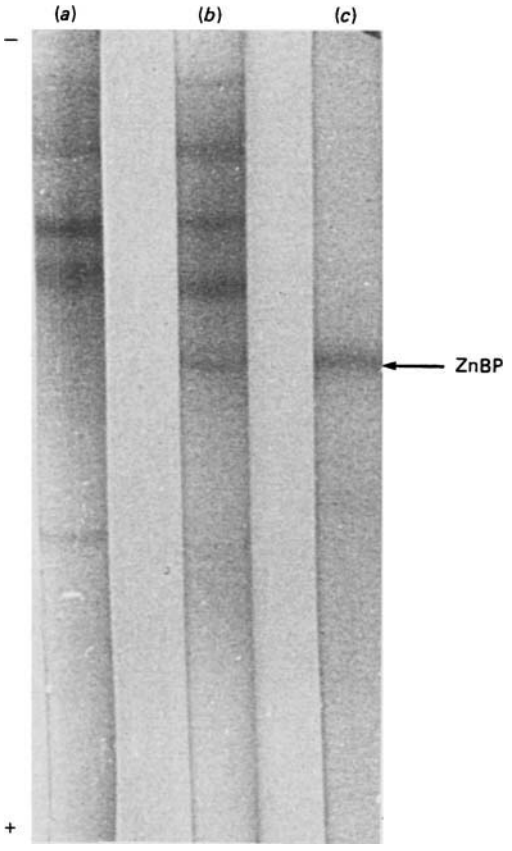

(*Facing p.* 268)

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