Review

Differential Regulation of Calbindin in the Calcium-Transporting Organs of Birds with High Calcium Requirements

Arie Bar

Institute of Animal Science, ARO, the Volcani Ctr., Bet Dagan, Israel

The present review focuses on the differential regulation and differential expression, as well as the possible differential functionality, of avian calbindin-D\(_{28k}\) in the three major calcium-transporting tissues: intestine, eggshell gland (ESG) and kidney. Special emphasis is given to their relationships with fast growth and with the laying of long clutches, i.e., unbroken sequences of eggs, separated by one or more days, as affected by the three major regulators, cholecalciferol (vitamin D\(_3\)), gonadal activity, and intra- or extracellular calcium or its fluxes. The accumulated evidence suggests that intestinal calbindin mostly depends on vitamin D\(_3\), ESG calbindin appears to depend on calcium-transport-related factor(s) and to lesser extent also on gonadal activity; whereas renal calbindin partly depends on vitamin D\(_3\) and extracellular calcium.

Key words: age, calbindin, calcium binding protein, cholecalciferol (vitamin D\(_3\)), dietary alteration, eggshell gland, growth, intestine, kidney, laying, maturation


1. Introduction and General Considerations

The mechanisms involved in calcium transport in the intestine and, to a lesser extent, in the other calcium-transporting organs such as the kidney, placenta, mammalian uterus and avian eggshell gland (uterus, ESG) have been intensively studied since the 3\(^{rd}\) decade of the 20\(^{th}\) century (Stewart and Percival, 1927; Nicolaysen, 1937; Migicovsky and Nielson, 1951; Schachter and Rosen, 1959 and many others\(^1\)). Although the importance of vitamin D in the intestinal calcium transport mechanism was already accepted by that time, the involvement of calcium binding/transporting proteins in calcium transport in the chick and rat intestine were demonstrated only in 1966 and 1967, respectively (Wasserman and Taylor, 1966; Kallfelz et al., 1967). Later, these proteins were identified as calbindin-D\(_{28k}\) and calbindin-D\(_{31k}\), respectively (see 1.1), and were identified in many other species and many other calcium-transporting tissues, and also in other tissues. Calbindin-D\(_{28k}\) is found almost only in mammalian tissues, whereas calbindin-D\(_{31k}\) is found in tissues of birds and lower vertebrates, and in mammalian kidneys and nervous tissues (see 1.3). These proteins were intensively studied with regard to their involvement in calcium transport in avian and mammalian species (see 1.4).

1.1. Biochemical and Molecular Characteristics of Calbindin-D\(_{28k}\)

The biochemical, molecular and physiological characteristics of calbindins have been widely reviewed (Thomasset, 1997; Christakos et al., 2003; Choi et al., 2005; Christakos et al., 2005), as has their involvement in avian egg-laying status and shell calcification (Bar, 2008).

Calbindins are a group of proteins belongs to a subfamily of calcium-binding proteins containing EF-hand structures. Their EF-domains consist of about 28 amino acids and have a helix-loop-helix structure that selectively binds ionic calcium (Ca\(^{2+}\)). The expression of this group of proteins, previously named CaBPs (calcium-binding proteins), differs (Table 1) among many cell types and species. Avian intestinal, ESG and kidney calbindins are identical, having the same molecular weight, immunogenicity and amino acid composition (Taylor and Wasserman, 1972; Bar et al., 1976a; Fullmer et al., 1976). The amino acid sequence of calbindin-D\(_{28k}\) was established by chemical mapping (Fullmer and Wasserman, 1987) and from the complementary DNA sequence (Wilson et al., 1985; Hunziker, 1986); it consists of 261 amino acids and contains six EF hands, four of which bind Ca\(^{2+}\) strongly. The apparent \(K_a\) is \(10^8\) M\(^{-1}\) to \(10^6\) M\(^{-1}\). Lower affinity was observed for other cations, diminishing in the

\(^1\) Only few of the relevant reviews or original articles are cited. Efforts have been made in most cases to cite the most recent reviews and the earlier original papers. In some cases other (more recent, more relevant or more comprehensive) publications are cited.
### Table 1. Differential regulation of avian calbindin: effects of selected physiological and nutritional alterations

<table>
<thead>
<tr>
<th>Cause/tissue</th>
<th>Intestine</th>
<th>Kidney</th>
<th>ESG</th>
<th>References(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D derivatives in vitamin-D-deficient(^2)</td>
<td>+ + + (^1)</td>
<td>+</td>
<td>=/+ (^4)</td>
<td>(Wasserman and Taylor, 1966; Corradino et al., 1968; Taylor and Wasserman, 1972; Bar and Wasserman, 1974)</td>
</tr>
<tr>
<td>1-hydroxylated derivatives in vitamin-D-fed</td>
<td>+ + +</td>
<td>=</td>
<td>=</td>
<td>(Wasserman and Taylor, 1973; Bar et al., 1975; Bar et al., 1976b)</td>
</tr>
<tr>
<td>Dietary Ca(^{2+}) restriction in vitamin-D-fed</td>
<td>+ + +</td>
<td>=/−</td>
<td>=/−</td>
<td>(Wasserman and Taylor, 1972, 1973c; Bar et al., 1975; Bar et al., 1978b)</td>
</tr>
<tr>
<td>Dietary P restriction in vitamin-D-fed</td>
<td>+ + +</td>
<td>+++</td>
<td>=</td>
<td>(Morrissey and Wasserman, 1971; Bar and Wasserman, 1973; Bar et al., 1975; Bar and Hurwitz, 1984)</td>
</tr>
<tr>
<td>Dietary Ca(^{2+}) restriction in 1-hydroxylated-D-fed</td>
<td>=</td>
<td>=</td>
<td>ND(^3)</td>
<td>(Bar and Wasserman, 1973; Bar et al., 1975)</td>
</tr>
<tr>
<td>Dietary P restriction in 1-hydroxylated-D-fed</td>
<td>+ + +</td>
<td>+++</td>
<td>ND</td>
<td>(Bar and Wasserman, 1973; Bar et al., 1975)</td>
</tr>
<tr>
<td>High dietary Ca(^{2+}) in vitamin-D-fed</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>(Bar et al., 1990a)</td>
</tr>
<tr>
<td>Growth</td>
<td>+ +</td>
<td>=</td>
<td>ND</td>
<td>(Bar and Hurwitz, 1981)</td>
</tr>
<tr>
<td>Maturation or gonadal hormones</td>
<td>+ +</td>
<td>=</td>
<td>=/+</td>
<td>(Bar and Hurwitz, 1972, 1973c; Montecucchi et al., 1977b; Bar et al., 1978b; Bar and Hurwitz, 1979b; Navickis et al., 1979a, b)</td>
</tr>
<tr>
<td>Laying</td>
<td>+ + +</td>
<td>=</td>
<td>+++</td>
<td>(Corradino et al., 1968; Wasserman and Taylor, 1968; Bar and Hurwitz, 1972, 1973c; Bar et al., 1978b)</td>
</tr>
<tr>
<td>Shell calcification (on protein synthesis)</td>
<td>=</td>
<td>ND</td>
<td>=</td>
<td>(Bar and Hurwitz, 1975)</td>
</tr>
<tr>
<td>Shell calcification (on mRNA synthesis)</td>
<td>=</td>
<td>ND</td>
<td>+++</td>
<td>(Nys et al., 1989; Striem and Bar, 1991; Bar et al., 1992a; Nys et al., 1992a)</td>
</tr>
</tbody>
</table>

\(^1\) The table tries to bring together most earlier published quantitative or semi-quantitative comparisons, as well as supporting evidence published later by other research teams. Many, but not all, other studies are mentioned in the text and in the References list. Few of the very early studies used the chelalex assay, but their findings were confirmed later with immunoassays or Western analysis; with permission from (Bar, 2008).

\(^2\) Not laying.

\(^3\) The regulative response varied between none (=) to very strong (+ + +) or negative (−); ND, not detected.

\(^4\) Varied in accordance with laying rate.

\(^5\) Not determined.

Order Ca\(^{2+}\) > Cd\(^{2+}\) > Sr\(^{2+}\) > Mn\(^{2+}\) > Zn\(^{2+}\) > Ba\(^{2+}\) > Co\(^{2+}\) > Mg\(^{2+}\) (Ingersoll and Wasserman, 1971). The protein has a high content (30%) of dicarboxylic amino acids, many polar residues, an isoelectric point of pH 4.2–4.3 and a slight biphatic pH optimum for Ca\(^{2+}\) binding (reviewed in Wasserman et al., 1992; Christakos et al., 2003; Christakos et al., 2005). The calbindin-D\(_{28k}\) gene is about 19 kb long, and is highly conserved during evolution (5.4% change per 100 million years). The avian protein consists of 11 exons (Minghetti et al., 1988; Wilson et al., 1988). The coding region exhibits 79% homology with the rat brain calbindin-D\(_{28k}\) (nucleotides 286–1068; Hunziker, 1986). A putative vitamin-D-responsive element (VDRE) was identified on the mouse calbindin-D\(_{28k}\) promoter. Some observations also indicate the presence of putative low-active or inactive VDREs on the chicken gene, encoding calbindin-D\(_{28k}\) (reviewed in DeLuca, 2004; Christakos et al., 2005). In addition, an estrogen-responsive element was identified on the 5′-flanking region of the mammalian calbindin-D\(_{28k}\) promoter (Gill and Christakos, 1995; Criddle et al., 1997). At least in the avian intestine, kidney, ESG and brain, three species of mRNAs are encoded: a major (approximately 2.0 kb) and two minor species (approximately 2.7 and 3.0 kb, Fig. 1 a-d) all encode calbindin-D\(_{28k}\). Intestinal and renal calbindin mRNAs, but not those of the ESG (because of its oscillations during the diurnal egg cycle; see 2.2.3.), were closely correlated (Bar et al., 1990a) with the
calbindin concentration in the same tissues; covariance analysis indicated that the slopes and intercepts were not significantly different. This suggests that most of the changes in intestinal and renal calbindin may be attributed to changes in the mRNA. This suggestion is consistent with other observations (Huang and Christakos, 1988; Fullmer, 1990).

1.2. Determination of Avian Calbindin

Most earlier studies employed the Chelex ion-exchange resin assay to determine the Ca$^{2+}$ binding capacity of soluble compounds in the supernatants of the calcium-transporting organs (Wasserman and Taylor, 1966). Heat treatment, dialysis, salting and desalting, Sephadex G-25 column fractionation as well as trypsin digestion were used in order to ensure that the soluble compounds really were proteins (Wasserman and Taylor, 1966; Taylor and Wasserman, 1969; Bar and Hurwitz, 1972). This method was adopted by many other researchers (Hurwitz and Bar, 1969; Bar and Hurwitz, 1972; Freund and Bronner, 1975; Thomasset et al., 1976; Navickis et al., 1979b). The absence of this protein from the intestine of the vitamin D-depleted chick enables use of Western blot analysis to
obtain clear visualization of the specific band of calbindin-D$_{28k}$ in the intestine of cholecalciferol (vitamin D$_{3}$)-treated birds. The migration of this band on a 10–15% polyacrylamide gel without dodecyl sulfate (SDS) is much faster than that of any other band, therefore it could be easily identified (Fig 2. and Wasserman and Taylor, 1966; Taylor and Wasserman, 1969; Bar and Hurwitz, 1972).

The purification of calbindin-D$_{28k}$ (Wasserman et al., 1968) and calbindin-D$_{28k}$ (Fullmer and Wasserman, 1973) enabled the production of polyclonal and, later, monoclonal antibodies that were used, first for the radial immunodiffusion assay (Corradino, 1973) or Rocket immunoelectrophoresis (Bar and Wasserman, 1974; Taylor, 1974), and subsequently for radioimmunoassay of avian calbindin-D$_{28k}$ and mammalian calbindin-D$_{28k}$ (Bar and Hurwitz, 1979b; Christakos et al., 1979; Marche et al., 1977, respectively). Recently, in order to avoid the use of radioisotopes, an ELISA assay for avian calbindin was developed (Yosefi et al., 2003). The availability of specific antibodies against avian calbindin also enables its use for immunohistochemical studies (Lippiello, 1974; Lippiello and Wasserman, 1975; Jande et al., 1981; Rhoten and Christakos, 1981; Wu et al., 1993, 1994; Sugiyama et al., 2007 and many others). These methods were widely applied in many other studies (not cited in detail). The availability of specific antibodies for calbindin-D$_{28k}$ enables its visualization also by Western blot analysis in the presence of SDS.

Continuing progress in molecular biology raised particular interest in regulating the expression of calbindin genes and in synthesizing their mRNA (Morrissey et al., 1978; Spencer et al., 1978; Christakos and Norman, 1980; Thomasset et al., 1981). Visualization of expression of the calbindin genes was first done by Northern blotting (King and Norman, 1986; Warembourg et al., 1986; Varghese et al., 1988) with $^{32}$P-radiolabeled cloned DNA or short (approximately 33 nucleotides) oligonucleotides (Bar et al., 1990a; Striem and Bar, 1991; Bar et al., 1992b, 1996).

Various densitometry methods were attempted for semi-quantitative evaluation of specific Northern bands (Warembourg et al., 1986; Varghese et al., 1988), and use of dot-blot assays was also examined (Warembourg et al., 1986; Armbricht et al., 1989). More quantitative evaluation was achieved by using filter hybridization, with a complementary RNA (Mayel-Afshar et al., 1988), or by means of the solution hybridization competitive binding assay that used complementary oligonucleotides (Bar et al., 1990a; Fullmer, 1990). Recently the real-time/polymerase chain reaction (RT-PCR) (Liu et al., 1996; Song and Fleet, 2004; van der Eerden et al., 2005) or the ribonuclease protection assay (RPA) (Armbricht et al., 1999) was also used for the detection of calbindin mRNAs, but these have not yet been applied to determination of avian calbindin in the three calcium transporting organs. The availability of specific probes for calbindin mRNA makes possible the histochemical determination of calbindin gene expression (Warembourg et al., 1986; Rhoten and Christakos, 1990; Wu et al., 1993; Lavelin et al., 1998).

1.3. Tissue Distribution and Localization

High concentrations of calbindins are found in tissues that are characterized by their massive transport of Ca$^{2+}$, such as the intestine, kidney, placenta, mammalian uterus and, in birds, the ESG (Wasserman and Taylor, 1966; Taylor and Wasserman, 1967; Corradino et al., 1968; Bruns et al., 1978; Marche et al., 1978; Delorme et al., 1983). Calbindin-D$_{28k}$ was found also in the avian chorioallantoic membrane (Ono and Tuan, 1991; Sechman et al., 1994). Low concentrations of calbindins are found in other tissues associated with Ca$^{2+}$ homeostasis and metabolism, such as bone, tooth cells and parathyroid cells. These proteins are also found in tissues not directly associated with Ca$^{2+}$ transport: the nervous tissues contain high concentrations, and the pancreas and testes contain low concentrations (reviewed in Thomasset, 1997; Christakos et al., 2005). Calbindin-D$_{28k}$ was also found in the intestines and kidneys of reptilian and amphibian species, and in the brains of many other vertebrates (Rhoten et al., 1984, 1986; Parmentier et al. 1987).

Whereas the mammalian intestine, uterus, placenta, and other tissues (including the kidney in a few species) contain mainly calbindin-D$_{28k}$, the nervous tissues and most mammals’ renal tissues contain calbindin-D$_{28k}$. The

---

2Ca-ATPase, carbonic anhydrase and a high-molecular-weight (approximately 100 kDa) calcium-binding protein were also detected in the chorioallantoic membrane (Tuan et al., 1986).
tissues of avians and other lower vertebrates contain almost exclusively calbindin-D$_{28k}$. The avian calbindin is similar, but not identical to the mammalian calbindin-D$_{28k}$ (Hunziker, 1986; Christakos et al., 1989). Minute concentrations of calbindin-D$_{9k}$ are also found in a few avian tissues (Zanello et al., 1995).

In the chick intestine and ESG, the protein is localized primarily in the absorptive cells and the tubular gland cells, respectively (Lippiello and Wasserman, 1975; Jande et al., 1981; Wasserman, et al., 1991). Renal calbindin-D$_{28k}$ is exclusively localized in the distal convoluted and collecting tubules of mammals (reviewed in Christakos et al., 2005; van de Graaf et al., 2007) and birds (Lippiello, 1974; Jande et al., 1981). In the chicken and quail intestine, calbindin concentrations are higher in the proximal than in the distal segments (Taylor and Wasserman, 1967; Bar et al., 1976b).

1.4. Biological Activity

In all three major Ca$^{2+}$-transporting organs, the intestine, the kidney and the ESG of birds, concentrations of calbindin are closely correlated with Ca$^{2+}$ transport (Figs. 3a, b and Taylor and Wasserman, 1969; Morrissey and Wasserman, 1971; Bar and Hurwitz, 1975, 1979b; Bar et al., 1984; Rosenberg et al., 1986; Bar et al., 1992b). However, whereas the calbindin contents in the kidney (Bar et al., 1975) and in the ESG (Bar et al., 1984, 1992b, 1999) are correlated with the mass of calcium transported (weight unit), the intestinal calbindin is correlated with calcium transport capability (absorption percentage), which is low in birds fed high dietary calcium, although the mass absorbed by these birds is high; Fig. 3c; (Bar et al., 1979). Furthermore, whereas none of the observed changes in intestinal calbindin and its mRNAs could be attributed to changes in plasma Ca$^{2+}$ content, renal calbindin mRNA and calbindin were positively related to plasma Ca$^{2+}$ in vivo (Taylor and Wasserman, 1972; Rosenberg et al., 1986; Bar et al., 1990a) or to extracellular calcium in tissue culture (Clemens et al., 1989).

In birds and mammals the overall Ca$^{2+}$ transport represents the sum of unsaturated and saturated processes. Whereas the unsaturated process consists of diffusion/movement through a regulated paracellular route that utilizes the tight junctions (TJs; which link epithelial cells and form a biological barrier) permeability for ions (reviewed in Schneeberger and Lynch, 2004; Van Itallie and Anderson, 2006), the saturated active transcellular

---

**Fig. 3.** Relationships between (a) intestinal calbindin (CaBP) and intestinal capability to absorb calcium, (b) between eggshell gland (ESG) calbindin and shell calcium in laying hens during periods of shell calcification, and (c) plasma calcium, intestinal and plasma calbindin (CaBP), and intestinal calcium absorption as functions of calcium intake. (a) $r = 0.930, P < 0.001$; non-laying, ○; laying hens during periods of shell calcification, ●. (b) $r = 0.854, P < 0.001$, respectively; with permission from (Bar and Hurwitz, 1979b), (Bar et al., 1984) and (Bar et al., 1979), respectively.
transport (reviewed in Bouillon et al., 2003; Bronner 2003; Hoenderop et al., 2005; Wasserman, 2005; van de Graaf et al., 2007) involves three major steps: entry of Ca\(^{2+}\) through the brush border, diffusion or movement to the basal membrane, and extrusion through the basal membrane. The first step proceeds down the chemical gradient of Ca\(^{2+}\) that results from its low concentration (10\(^{-7}\)M) in cells and high concentrations (>10\(^{-3}\)M) in the plasma or intestinal lumen. This step appears to be facilitated by epithelial calcium channels (TRPVs) (reviewed in Hoenderop et al., 2005; van de Graaf et al., 2007; Venkatachalam and Montell, 2007).

The second step appears to be facilitated by intracellular calbindins, which function either as transporter proteins (Feher, 1984; Koster et al., 1995) or as a buffer that regulates Ca\(^{2+}\) in close proximity to the TRPV pores by direct association with the channel (Lambers et al., 2006; Christakos et al., 2007; Schoeber et al., 2007). In addition, there is some evidence for calbindin-mediated cytosolic-free diffusion of Ca\(^{2+}\) and for vesicular transport of Ca\(^{2+}\) (reviewed in Norman et al., 2002; Larsson and Nemere, 2003; Dusso et al., 2005).

Besides the suggested role of calbindins in Ca\(^{2+}\) transport, they may also be involved in protecting the cells from high concentrations of Ca\(^{2+}\), or from cellular degradation via apoptosis (Christakos et al., 2003).

The energy-dependent third step proceeds up the chemical gradient of Ca\(^{2+}\) that results from the low cellular Ca\(^{2+}\) concentration and the higher plasma (1.25 to 1.50 mM) and ESG luminal Ca\(^{2+}\) concentrations. This step is facilitated by the plasma membrane calcium-ATPase (PMCA) and, to a lesser extent, by a sodium-calcium (Na\(^{+}\)/Ca\(^{2+}\)) exchanger (reviewed in Hoenderop et al., 2005; Lytton, 2007).

### 2. Differential Regulation

Despite the chemical and apparent functional similarities, the synthesis of avian calbindin is differentially regulated in the three major transporting organs: intestine, ESG and kidney\(^1\). There is also evidence to suggest that calbindin, most likely, functions differently in these three organs.

#### 2.1. Vitamin D and Dietary Alterations

##### 2.1.1. Intestine

Levels of calbindin-D\(_\text{28k}\) and its mRNA in the chick intestine were found to be very low before hatching but were significantly increased on days 1 to 7 after hatching (Taylor and Wasserman, 1972; Opperman et al., 1990; Sechman et al., 1994). However, two days before hatching the embryonic intestine in vitro is already responsive to vitamin D metabolites and is capable of synthesizing calbindin and its mRNAs (Corradino, 1973; Corradino and Fullmer, 1991). Post hatching, 1,25(OH\(_3\))D\(_3\) (the active hormonal form of vitamin D\(_3\), reviewed in Dusso et al., 2005; Norman, 2006; Christakos et al., 2007; Bar, 2008; and many others) induces the formation of all three species of mRNA (Fig. 1a; see also 1.1) in the avian intestine and kidney, but not in the ESG (Hunziker, 1986; Mayel-Afshar et al., 1988; Bar et al., 1990a; Striem and Bar, 1991; Bar et al., 1992a). Levels of intestinal calbindin mRNAs and of calbindin synthesis reflect the changes in 1,25(OH\(_2\))D\(_3\) in the blood and the intestinal cells (Friedlander et al., 1977; Bar et al., 1982; Hunziker et al., 1982; Striem, 1990). These include the changes that result from exogenous supplementation of vitamin D or its derivatives (Wasserman and Taylor, 1966; Bar et al., 1975; Edelstein et al., 1975; Bar et al., 1976b; Friedlander et al., 1977; Swaminathan et al., 1977; Bar et al., 1978b, 1990b; Striem and Bar, 1991; Bar et al., 1992a) as well as those formed endogenously (reviewed in Bar, 2008) in response to dietary Ca\(^{2+}\) and/or phosphorus (P) alterations. Restrictions of dietary Ca\(^{2+}\) and/or P stimulate the synthesis of intestinal calbindin mRNAs and/or calbindin in both non-laying (Wasserman and Taylor, 1968; Morrissey and Wasserman, 1971; Bar et al., 1972; Friedlander et al., 1977; Montecuccoli et al., 1977a; Swaminathan et al., 1977; Bar et al., 1990a) and laying birds (Bar and Hurwitz, 1973b, 1984; Nys et al., 1992a; Bar et al., 1999), as well as in mammals (Freund and Bronner, 1975; Thomasset et al., 1976; Armbrrecht et al., 1980b; Sommerfeve et al., 1985). Dietary excess of Ca\(^{2+}\) did not affect the contents of calbindin mRNAs and/or calbindin, or slightly reduced them, whereas dietary excess of P did not affect intestinal calbindin content (Morrissey and Wasserman, 1971; Bar et al., 1979; Hurwitz et al., 1995; Bar et al., 2003). Of interest is the finding that restriction of dietary P but not of Ca\(^{2+}\) induced intestinal Ca\(^{2+}\) absorption and calbindin synthesis in birds supplemented only with 1-hydroxylated derivatives of vitamin D (Bar and Wasserman, 1973; Bar et al., 1975). This suggests that the effect of dietary P is not fully dependent on the renal 1-hydroxylation of vitamin D (reviewed in Bar, 2008). A similar effect on intestinal calbindin-D\(_{28k}\) and intestinal calcium absorption was observed also in 1,25(OH\(_2\))D\(_3\)-fed rats (Ribovich and DeLuca, 1975; Thomasset et al., 1977).

The increase in 1,25(OH\(_2\))D\(_3\) (reviewed in Wasserman, 2005; Bar, 2008 and many others) induced calbindin, an important component of the transcellular transport mechanism, synthesis in P-restricted animals appears to be an anti-homeostatic response (Morrissey and Wasserman, 1971; Bar and Wasserman, 1973; Montecuccoli et al., 1977a; Bar et al., 1978b; Bar and Hurwitz, 1979a; Bar et al., 1990a), as it accelerates the severe hypercalcemia that develops under this nutritional condition.

Other nutritional factors or chemicals such as strontium (Corradino et al., 1971; Omdahl and DeLuca, 1972; Armbrecht et al., 1980a), aluminum (Dunn et al., 1993), lead (Fullmer and Rosen, 1990; Cox and Dunn, 2001), biphosphonates (EHDP) and lathyrogens (Bar and

---

\(^1\)The present review focuses on intestinal, renal and ESG calbindin-D\(_{28k}\). Other calbindin-containing tissues, including the brain, that contain vitamin D-independent calbindin, are not addressed here.
Hurtwitz, 1973a, 1974) have been found also to affect intestinal calbindin. Several other factors, such as cadmium or zinc, inhibited calbindin-D<sub>28K</sub> synthesis in the embryonic intestine in vitro using the tissue culture technique (Corradino and Fullmer, 1980). The activities or modes of action of these factors are not addressed in this review; however some of these factors, such as strontium, appear also to affect calbindin synthesis, through their effects on vitamin D metabolism (Omdahl and DeLuca, 1972).

Physiological conditions associated with alterations in vitamin D metabolism (reviewed in Bar, 2008) such as growth rate, breed, age, energy intake (Bar and Hurtwitz, 1981; Bar et al., 1988; Hurtwitz et al., 1995; Bar et al., 1999, 2003; Yosefi et al., 2003) or gender (Song and Fleet, 2004 in mammals), maturation and onset of laying are also associated with increased synthesis of calbindin mRNAs and/or calbindin, whereas arrest of laying is associated with reductions in their synthesis (for details see 2.2.3. and Wasserman and Taylor, 1968; Bar and Hurtwitz, 1972; Striem and Bar, 1991; Bar et al., 1992a; Nys et al., 1992a; Sugiyama et al., 2007).

The age of the laying hen did not affect intestinal calbindin synthesis, or slightly (not significantly) reduced it. This slight reduction may be from the result of the marked drop in calcium needs (Fig. 4). However, the aged laying hen loses some of its ability to adapt, via vitamin D metabolism, to changes in calcium intake or needs (Bar and Hurtwitz, 1987; Bar et al., 1999). In the rat, age (Armbrecht et al., 1989) did affect intestinal calbindin and, in addition, an age-dependent loss of adaptive capability was also observed (Armbrecht et al., 1980 b). The effects of dietary Ca<sup>2+</sup> or P restrictions (Freund and Bronner, 1975; Thomasset et al., 1976, 1977) and of growth hormone (Bruns et al., 1983), pregnancy and lactation (Bruns et al., 1987; Zhu et al., 1998) on intestinal calbindin-D<sub>28K</sub> were also observed in mammals.

Although there has not yet been a comprehensive comparative study, there is some evidence to suggest that laying breeds may also differ in their intestinal calbindin contents (Yosefi et al., 2003).

A single observation (Shirley et al., 2003) indicated that there was a reduction in intestinal calbindin content in vitamin D-restricted (but not deficient) chicks that had been selected for high incidence of tibial dyschondroplasia (TD). As 1-hydroxylated derivatives of vitamin D<sub>3</sub> are known to reduce TD incidence and severity (reviewed in Whitehead, 1998; Edwards, 2000), this finding could be an indication of a vitamin D-dependent involvement of calbindin in the development of the syndrome.

Vitamin D dependency of intestinal calbindin in birds was observed in a variety of species, such as the domestic hen (Gallus domesticus) (Wasserman and Taylor, 1966), the Japanese quail (Coturnix coturnix japonica) (Bar et al., 1976a; Musser et al., 1977), the turkey (Meleagris gallopava) (Hurtwitz et al., 1973; Musser et al., 1977; Bar et al., 1978b) and the duck (Anas platyrhynchos) (Bar et al., unpublished results). Calbindin was also found in the ostrich (Struthio camelus) intestine (Fig. 2. and Bar et al., unpublished results).

2.1.2. Kidney

In non-mammals, as well as in most mammals, the kidney contains almost exclusively calbindin-D<sub>28K</sub> (reviewed in Christakos et al., 2005). In the chick embryo, renal calbindin is high and was detected already at the 7<sup>th</sup> embryonic day (7E). After 10E to 12E, renal calbindin-D<sub>28K</sub> and its mRNA appear to diminish gradually until hatching (Taylor and Wasserman, 1972; Opperman...
as in the intestine, the full modulation of renal calbindin and its mRNAs, requires vitamin D metabolites, without which levels of renal calbindin and its mRNAs are very low and are almost unaffected by dietary alterations. However, unlike intestinal calbindin, the renal calbindin, although significantly diminished in vitamin D-deficient chicks, did not completely disappear, and even retained part of its susceptibility to modulation in response to dietary alteration. Furthermore, unlike the intestinal calbindin, the renal calbindin is not fully correlated with plasma or renal $1,25(\text{OH})_2\text{D}_3$ (Bar et al., 1975; Rosenberg et al., 1986; Clemens et al., 1988; Bar et al., 1990a; Hall and Norman, 1990): under some nutritional or physiological conditions such as calcium deficiency or the onset of egg formation, plasma $1,25(\text{OH})_2\text{D}_3$ increased but renal calbindin did not (Table 1 and Bar et al., 1975; Edelstein et al., 1975; Bar et al., 1978b; Bar and Norman, 1981; Rosenberg et al., 1986).

Dietary P, but not dietary calcium, restriction also markedly induces the synthesis of renal, but not ESG (Bar and Hurwitz, 1984) calbindin-$28k$ in both non-laying (Bar et al., 1975; Rosenberg et al., 1986; Bar et al., 1990a) and laying (Bar and Hurwitz, 1984) birds, and in mammals (Thomasset et al., 1977; Huang and Christakos, 1988; Varghese et al., 1988). The increased renal calbindin synthesis in P-restricted birds seems to add support to the hypothesis that renal calbindin is regulated by $1,25(\text{OH})_2\text{D}_3$, as the levels of the hormone in the tissue are elevated in vitamin D$_2$-fed, P-restricted chicks (Edelstein et al., 1975; Baxter and DeLuca, 1976; Friedlander et al., 1977; Rosenberg et al., 1986). However, there is considerable evidence against this hypothesis: renal 1-hydroxylase activity is markedly enhanced in response to dietary Ca$^{2+}$ restriction, and was diminished in birds fed Ca$^{2+}$-rich diets (Henry et al., 1974; Montecuccoli et al., 1977a; Bar et al., 1978c, 1990a). These changes, and the consequent changes in the plasma and intestinal concentrations of $1,25(\text{OH})_2\text{D}_3$, may account for the observed increases in intestinal calbindin and its mRNAs in the vitamin D$_2$-fed chicks. However, renal calbindin remained unchanged, or even reduced, in Ca$^{2+}$-restricted birds, despite the increases in plasma and renal $1,25(\text{OH})_2\text{D}_3$ contents (Rosenberg et al., 1986). Furthermore, the increased renal calbindin in P-deficient chicks occurred also (in most studies) when the vitamin D-1-$\alpha$-hydroxylase regulatory mechanism was bypassed by the use of 1-hydroxylated derivatives of vitamin D (Bar and Wasserman, 1973; Bar et al., 1975; Rosenberg et al., 1986; Bar et al., 1990a).

Whereas dietary calcium restriction slightly reduced renal calbindin mRNAs and calbindin, high dietary calcium induced their renal synthesis, but to a lesser extent than was observed in P-restricted birds. None of the observed changes in intestinal calbindin and its mRNAs could be attributed to changes in plasma Ca$^{2+}$ content, but renal calbindin mRNA and calbindin were positively related to plasma Ca$^{2+}$ (Taylor and Wasserman, 1972; Rosenberg et al., 1986; Bar et al., 1990a) and to urinary Ca$^{2+}$ excretion, independently of the bird’s vitamin D status (Bar et al., 1975; Clemens et al., 1989; Bar et al., 1990a). It appears, therefore, that extracellular Ca$^{2+}$ plays a major role in the modulation of renal calbindin synthesis.

The mode of action of renal calbindin has been studied mostly in mammals. Mammalian calbindin-D$_{28k}$, similarly to TRPV5, is localized exclusively in the distal convoluted and collecting tubules, where only a minor proportion of the Ca$^{2+}$ reabsorption occurs. A recent hypothesis (reviewed in van de Graaf et al., 2007) suggests that the majority of Ca$^{2+}$ reabsorption occurs passively in the proximal tubules whereas the thick ascending loop of Henle is responsible for 20% of the Ca$^{2+}$ reabsorption, most likely via a regulated paracellular mechanism, and only 15% of the filtrates is reabsorbed in the posterior part of the distal convoluted and the collecting tubules, via a transcellular mechanism associated with calbindin, TRPV5 and other proteins. Even if the above hypothesis is valid in birds also, it still would not explain the positive relationship between extracellular, or urinary Ca$^{2+}$ and renal calbindin, which occurs under dietary P restriction (Bar; Rosenberg; Bar a) and to urinary $1,25(\text{OH})_2\text{D}_3$, as the levels of the hormone in the tissue animals, as well as its possible action as a buffer protein that prevents excessive cellular Ca$^{2+}$, or as an anti-apoptotic factor (Christakos et al., 2003) should be considered.

2. I. 3. Eggshell gland

Most of the available evidence does not support the idea that ESG calbindin-$28k$ is vitamin D dependent: endogenous $1,25(\text{OH})_2\text{D}_3$ or exogenous 1-hydroxylated vitamin D$_3$ derivatives (Bar et al., 1976b, 1988, 1990b) had no effect on ESG calbindin, whereas it did affect intestinal or renal calbindin. Furthermore, ESG calbindin mRNAs were induced in shell-forming, vitamin D-deficient quail that were maintained on a high-Ca$^{2+}$ diet to promote their laying (Striem and Bar, 1991). Moreover, dietary calcium alterations that modulated intestinal and renal calbindin, or dietary P restriction that increased intestinal and renal calbindin in accordance with their effects on vitamin D metabolism, did not affect synthesis of ESG calbindin or its mRNAs (Bar and Hurwitz, 1973c; Bar et al., 1978b; Bar and Hurwitz, 1984; Bar et al., 1984, 1999; Ieda et al., 1999). This occurred despite the elevated plasma $1,25(\text{OH})_2\text{D}_3$ levels observed under dietary calcium or P restriction (Bar et al., 1984) or $1,25(\text{OH})_2\text{D}_3$ feeding (Bar et al., 1990b) and in spite of the elevated ESG $1,25(\text{OH})_2\text{D}_3$ observed in calcium restricted laying birds. Similarly, maturation prior to the onset of laying, which is associated with increased renal formation of $1,25(\text{OH})_2\text{D}_3$ (Montecuccoli et al., 1977b) and increased plasma and intestinal $1,25(\text{OH})_2\text{D}_3$, but not ESG $1,25(\text{OH})_2\text{D}_3$ (Bar et al., 1990a).
and Norman, 1981), is also associated with increases in intestinal calbindin and its mRNAs, but not in those of the ESG (Bar and Hurwitz, 1973c; Bar et al., 1978b, 1990b; Striem and Bar, 1991; Bar et al., 1992a, c; Wu et al., 1993; Bar et al., 1999; Goto et al., 2002b) or kidneys (Bar et al., 1978b).

On the other hand, the presence of a considerable concentration of VDR (20 to 33% of the intestinal concentration) in the ESG (Coty, 1980; Bar et al., 1984), as well as the parallel fluctuations of VDR (Ieda et al., 1995) and calbindin mRNAs in the ESG (Fig. 1b) during the egg cycle (Bar and Hurwitz, 1973c; Bar et al., 1978a, b; Nys et al., 1989; Bar et al., 1990b; Striem and Bar, 1991; Bar et al., 1992a, b; Nys et al., 1992b; Ieda et al., 1995), are indicative of a possible involvement of 1,25(OH)2D3 in ESG functionality (Bar et al., 1984, 1990b). This idea is further supported by the single finding that injection of 1,25(OH)2D3 directly into the ESG lumen increased the ESG calbindin concentration (Ohira et al., 1998); it is also supported by the finding that 1,25(OH)2D3 had a slight effect on synthesis of ESG calbindin mRNA in vitro, or of calbindin in vivo in estrogen-treated immature female chicks (Corradino, 1993; Corradino et al., 1993). These findings raised the question of whether the net accumulation of calbindin under these conditions (Bar and Hurwitz, 1973c; Bar et al., 1978b; Bar et al., 1984; Bar et al., 1992a; Bar et al., 1992b; Nys et al., 1992b; Bar et al., 1999; Ieda et al., 1999) was the combined result of enhanced vitamin D-dependent calbindin formation and, on the other hand, a parallel reduction in its synthesis caused by other factor(s). Such a combined effect might occur in the calcium-deficient bird, in which a possible increase in 1,25(OH)2D3-dependent synthesis of ESG calbindin might be nullified by a Ca2+-transport-dependent (see 2.2.3) reduction in calbindin synthesis as a result of the lower deposition of shell Ca2+. The overall result of such a combined effect might be nil or too small to be detected. Another possible explanation for the lack of effect of vitamin D on ESG calbindin could lie in the possibly limited capability of the tissue to synthesize calbindin and the fact that at the onset of egg formation the ESG reached its maximal capacity for such synthesis. This hypothesis is supported by the low VDR content in the ESG and by the finding that the calbindin level in the ESG of the high-producing layers (chicken and quail) was the highest level observed experimentally. This issue will be addressed further, in subsections 2.2.3 and 3.2.3.

2.1.4 Bone and other tissues

Vitamin D was found to affect calbindin-D28k, in the bone, pancreas and testes, but not in the nervous system (reviewed in Christakos et al., 2005). Calbindin-D28k synthesis in the mammalian pituitary, uterus and placenta, unlike that in the intestine and kidney, are also not stimulated by vitamin D or its metabolites (reviewed in Thomasset, 1997; Christakos et al., 2005; Choi and Jeung, 2008). On the other hand, uterine calbindin-D28k in mammals appears to be stimulated by estrogens (Bruns et al., 1988; Lhorset et al., 1990), and recent evidence suggests that placental calbindin is also regulated by estrogens (reviewed in Choi and Jeung, 2008). Among the other tissues the bone is of high importance for calcium homeostasis, but only little evidence is available concerning the distribution or functionality of bone calbindins (reviewed in Christakos et al., 2005). Calbindin-D9k (Balmain et al., 1986b; Berdal et al., 1996) was identified in mammalian bones, whereas calbindin-D28k was identified in both avian (Zhou et al., 1986) and mammalian (Balmain et al., 1986a; Berdal et al., 1996) bone tissues. In mammals, calbindin-D9k and calbindin-D28k, or their mRNAs, were identified in osteoblastic cells (Balmain et al., 1986a; Bellido et al., 2000). There is little evidence (van der Eerden et al., 2005) for their presence in osteoclasts. In birds, calbindin-D28k was found only in chondrocytes of growth plate cartilage (Zhou et al., 1986). The vitamin D dependency of bone calbindins is not yet established, although there is some evidence for such dependency in rodent teeth (reviewed in Christakos et al., 2005). The presence of VDRs in the bone cells (Kream et al., 1977; Chen et al., 1979 and many others) hints at a possible involvement of 1,25(OH)2D3 in the regulation of calbindin as well as in other vitamin-D-dependent proteins present in bone cells. In light of studies of mammalian bone cells, it was suggested that calbindins may be involved in protecting these cells from apoptosis (reviewed in Christakos et al., 2005) and/or in initiating bone mineralization (reviewed in Balmain, 1991) and/or that calbindins might play a part in the capability of osteoclasts to resorb calcium via the transcellular transport route that involves calbindin, TRPV5, Na+/Ca2+ exchanger 1, and plasma membrane Ca2+-ATPase 1b (van der Eerden et al., 2005), all of which are expressed in these cells.

Little information was obtained from studies with avian bone cells (reviewed in Sugiyama and Kusuhara, 2001). However, if the last two tentative hypotheses mentioned above are valid, then avian osteoblast and/or osteoclast (if it occurs there) calbindin may have a role in the remodeling of medullary bone during the egg cycle. This process accounts for the deposition of 25 to 40% of the shell calcium (reviewed in Bar, 2008), therefore the validation of these hypothesis could help to extend our understanding of the mechanisms of shell calcification.

2.2 Reproduction, Laying and Shell Calcification

2.2.1 Intestine

In the female bird intestinal calbindin mRNA (Striem and Bar, 1991; Bar et al., 1992a) and calbindin (Bar and Hurwitz, 1972; Montecuccoli et al., 1977b; Bar et al., 1978a; Bar and Norman, 1981; Bar et al., 1990b, 1992a; Nys et al., 1992a; Wu et al., 1994; Bar et al., 1996) are moderately increased during sexual maturation. Onset of laying markedly increased synthesis of calbindin mRNA (Fig. 1a and Nys et al., 1989; Bar et al., 1990b; Striem and Bar, 1991; Bar et al., 1992a; Nys et al., 1992a) and calbindin (Wasserman and Taylor, 1968; Bar and Hurwitz, 1973c; Bar et al., 1976a, 1978a, b; 1992a;
increased demand for calcium (reviewed in Bar, 1998). These findings suggest that estrogens alone cannot act
fed female chicks, most likely indirectly as a result of the induced by shell calcification in the normal laying bird.
Bar, Striem, Nys a) in vitamin-D- was one to two orders of magnitude smaller than that
mRNAs, in response to maturation or estrogen treatment
mimicked the effect of maturation on intestinal calbindin maximal increment in synthesis of ESG calbindin or its
formed uncalcified shells (Bar a, b; Nys ; Corradino ; Bar ) found that
thick shells, and was markedly lower or absent in hens that b; Striem, ; Nys b; Corradino,
hens that formed thin eggshells, intestinal calbindin was (reviewed in Choi ) most studies (Navickis
premolt or a slightly lower level (Yosefi ). In ble effect of estrogens on mammalian uterine calbindin
motion (Bar and Hurwitz, b, c; Bar a), molt induction (Yosefi ). Unlike intestinal
calbindin-D synthesis in the avian ESG was only slightly
calbindin-D synthesis in the avian ESG was only slightly
rat renal calbindin-D has responded to estrogen (Criddle et al., 1997), and the mouse calbindin-D promoter con-
tained several imperfect estrogen-responsive elements (Gill and Christakos, 1995).

2.2.2. Kidney
None of the reproductive factors, including maturation and laying (Bar et al., 1978b), that modulate intestinal or
ESG calbindin synthesis was found to affect renal calbindin synthesis in birds. The estrogen dependency of avian renal calbindin has not yet been determined, but the rat renal calbindin-D did respond to estrogen (Criddle et al., 1997), and the mouse calbindin-D promoter contained several imperfect estrogen-responsive elements (Gill and Christakos, 1995).

2.2.3. Eggshell gland
The ESG remains in a refractory state prior to actual reproduction, and calbindin mRNAs and calbindin begin to appear during calcification of the first eggshell (Bar and Hurwitz, 1973c; Bar et al., 1978a, b; 1990b; Striem and Bar, 1991; Bar et al., 1992a). During the diurnal egg cycle, calbindin mRNA contents in the ESG oscillated between near zero and high concentrations, in close temporal association with eggshell calcification (Fig. 1b, e and Bar et al., 1984; Rosenberg et al., 1986; Nys et al., 1989; Striem and Bar, 1991; Bar et al., 1992a, b; Nys et al., 1992 b; Ieda et al., 1995). Similarly to duodenal calbindin content, the ESG calbindin content was lower in non-
laying hens or in those that laid shell-less eggs than in those laying calcified eggs, and was lower in hens that
formed thin eggshells than in those that formed thick ones (Bar et al., 1984; Nys et al., 1986; Bar et al., 1988; Rabon et al., 1991; Bar et al., 1992a, b; Kang et al., 1996; Bar et al., 1999; Goto et al., 2002b). Similarly to the intestine, the ESG calbindin content was markedly decreased during molting and restored at the resumption of egg laying (Heryanto et al., 1997; Yosefi et al., 2003). Following molting, the ESG calbindin content was similar to or, in many but not all studies, slightly lower than that prior to molt induction (Yosefi et al., 2003). Unlike intestinal calbindin, ESG calbindin was not affected by the age at the onset of production (Bar and Hurwitz, 1973c; Bar et al., 1998).

The role of gonadal hormones in the development of the oviduct is well established. However, unlike the noticeable effect of estrogens on mammalian uterine calbindin (reviewed in Choi et al., 2005) most studies (Navickis et al., 1979b; Nys et al., 1989; Striem and Bar, 1989; Bar et al., 1990b; Striem, 1990; Nys et al., 1992b; Corradino, 1993; Corradino et al., 1993; Bar et al., 1996) found that calbindin-D synthesis in the avian ESG was only slightly induced by estrogens or sexual maturation. The observed maximal increment in synthesis of ESG calbindin or its mRNAs, in response to maturation or estrogen treatment was one to two orders of magnitude smaller than that induced by shell calcification in the normal laying bird. These findings suggest that estrogens alone cannot account for the markedly elevated synthesis of calbindin

Sugiyama et al., 2007) in the intestine and ESG. Early onset of egg production is associated with enhanced renal formation of 1,25 (OH)2D3 and higher intestinal content of calbindin than in hens that begin to lay later (Bar and Hurwitz, 1972; Bar et al., 1998). Molting (Yosefi et al., 2003), as well as any other factor that arrests egg production (Bar and Hurwitz, 1973b; c; Bar et al., 1992a), markedly reduces the intestinal calbindin content. During molt induced by fasting, intestinal calbindin disappeared and then reappeared, in accordance with the interruption and resumption of production (Fig. 5). During the post-
molt period intestinal calbindin content returned to the premolt or a slightly lower level (Yosefi et al., 2003). In hens that formed thin eggshells, intestinal calbindin was slightly lower (Bar et al., 1999) than in those that formed thick shells, and was markedly lower or absent in hens that formed uncalcified shells (Bar et al., 1992a, b; Nys et al., 1992a; Kang et al., 1996; Bar et al., 1999).

A combined treatment with estradiol and testosterone mimicked the effect of maturation on intestinal calbindin (Bar and Hurwitz, 1979b; Nys et al., 1984; Striem and Bar, 1989; Striem, 1990; Nys et al., 1992a) in vitamin-D-
fed female chicks, most likely indirectly as a result of the increased demand for calcium (reviewed in Bar, 2008, 2009) for medullary bone formation (reviewed in Dacke et al., 1993; Sugiyama and Kusuhara, 2001; Whitehead, 2004). Estrogen alone was found to induce calbindin synthesis in vitamin-D-treated chicks in only one study (Navickis et al., 1979a).

Fig. 5. The changes in body weight (BW), bone ash, plasma estradiol, ovary and oviduct weight, plasma calcium, duodenal and eggshell gland (ESG) calbindin during molt induction and recovery. Filled or open triangles and bars represent duodenal or ESG calbindin, respectively; with permission from (Yosefi et al., 2003).
mRNAs in the ESG of the shell-calcifying bird. On the other hand, the repetitive small effects of estrogens, together with the occurrence of an estrogen-responsive element on calbindin-D$_{283}$ promoter in the mouse and rat (Gill and Christakos, 1995; Criddle et al., 1997), support the idea that estrogens are involved in the mechanism of calbindin gene expression in the ESG, either directly, indirectly via their effects on oviduct development during maturation, or because of estradiol treatments (Striem et al., 1990; Corradino et al., 1993; Berg et al., 2001).

Whereas testosterone does not affect ESG calbindin synthesis, progesterone and dexamethasone inhibited it in vivo (Navickis et al., 1979a; Bar et al., 1996; Goto et al., 2002a), and progesterone did so in vitro also (Corradino, 1993). Dexamethasone, but not progesterone, also inhibited synthesis of intestinal calbindin and its mRNAs. Both prolonged the egg cycle, whereas the effect of testosterone remains open to question (Nys, 1987; Bar et al., 1996). Dexamethasone enhanced shell calcification, whereas progesterone inhibited Ca$^{2+}$ deposition onto the shell. These findings suggest that the effect of progesterone on ESG calbindin appears to be specific, and, as this effect is markedly more evident than the observed effects of estrogens on synthesis of calbindin mRNA and calbindin in the ESG, it appears that these effects are not due to the anti-estrogenic nature of progesterone. These results, together with the finding that plasma progesterone concentration oscillated during the diurnal egg cycle (Doi et al., 1980; Johnson and van Tienhoven, 1980; Braw-Tal et al., 2004) in close temporal association with eggshell calcification, and peaked about 4 h before the next ovulation (Bar, 2008) in coincidence with the diminution of shell calcification, provide further support for the hypothesis that progesterone has a specific and important role in shell calcification and ESG calbindin synthesis.

The absence of a major effect of vitamin D or estrogens on ESG calbindin, the association of ESG calbindin mRNAs but not of calbindin with shell formation, and the relationship of ESG calbindin with the mass of Ca$^{2+}$ transported rather than with the transport capability (as in the intestine), suggests that Ca$^{2+}$ transport in the ESG, similarly to that in the kidney, plays a major role in the synthesis of ESG calbindin mRNAs. In light of the above findings, it was hypothesized that the regulative mechanism for the synthesis of calbindin mRNAs in the ESG is a complex, multifactor process, which involves, in addition to a major Ca$^{2+}$-transport-related process, estrogen and other endocrine factors (Nys et al., 1989; Striem and Bar, 1991; Bar et al., 1992a; Nys et al., 1992b; Corradino, 1993; Corradino et al., 1993; Bar et al., 1996, 1999).

3. Conclusions

Calbindins are present in a variety of tissues in avian and mammalian, as well as in lower vertebrate species. Some of these tissues, including the intestine, kidney, avian ESG and mammalian placenta, uterus and mammary gland, are associated with calcium transport. The high affinity of these proteins for Ca$^{2+}$ qualifies them as components in the calcium-transport mechanism, either as transporter proteins that facilitate intracellular Ca$^{2+}$ diffusion or as buffer proteins that interact with Ca$^{2+}$ channels to prevent their inactivation by the accumulation of Ca$^{2+}$ in close proximity to their pores, and also as a buffer that prevents excessive cellular Ca$^{2+}$ levels, or as an anti-apoptotic factor. Despite the chemical similarity among the calbindins in the avian transporting tissues, i.e., the intestine, kidney and ESG, they appear to be differently regulated and, most likely, to play different roles in calcium transport in these respective tissues.

3.1. Intestine

Vitamin D is most likely the major regulator of intestinal calbindin synthesis. The actions of most of the other factors known to regulate intestinal calbindin synthesis, such as alterations in dietary calcium or P, age, growth, gender, breed, production, age at the onset of production, as well as many hormones, e.g., GH, estrogens, prolactin, could be attributed to their indirect effects on calcium requirements and, consequently, on vitamin D metabolism, i.e., the renal formation of 1,25(OH)$_2$D$_3$.

Most of the available data support the hypothesis that intestinal calbindin plays a major part in transcellular transport, either as an intracellular transporter protein that facilitates Ca$^{2+}$ diffusion toward the active Ca$^{2+}$ pump located at the basal membrane, and/or as a buffer that regulates Ca$^{2+}$ concentration in close proximity to the TRPV (Ca$^{2+}$ channels) pores and forms the chemical gradient required for TRPV functionality. This hypothesis is consistent with the positive correlations between calbindin content and the intestinal capacity to transport calcium, and the changes in plasma or intestinal 1,25(OH)$_2$D$_3$ contents, which occur even under conditions of dietary P restriction. However, dietary P restriction is characterized by increased Ca$^{2+}$ transport capability and increased Ca$^{2+}$ mass transport, although the latter represents an anti-homeostatic response associated with hypercalcemia that harms the birds. A part of the not yet precisely defined condition of the P-restriction (that is associated with increased intestinal content of calbindin in spite the high or normal dietary calcium), the transcellular transport involving calbindin, became the major mechanism only when the intake of Ca$^{2+}$ was lower than 0.6% of the diet (Bar, 2008, 2009).

3.2. Kidney

As in the intestine, full modulation of renal calbindin requires vitamin D metabolites, without which renal calbindin levels are very low and are almost unaffected by dietary alterations. Other factors, such as extracellular Ca$^{2+}$ or urinary calcium, may also be involved in the full regulation of renal calbindin. These factors account for the lack of association between 1,25(OH)$_2$D$_3$ and renal calbindin observed in P-restricted birds that were fed vitamin D or its 1-hydroxylated derivatives, or in birds fed high dietary calcium. The mechanism that enables the extracellular Ca$^{2+}$ to regulate renal calbindin synthesis is
yet to be elucidated.

Some of the observed findings (see 2.1.2) suggest that renal calbindin, similarly to intestinal calbindin, is involved solely in the transcellular Ca\(^{2+}\) transport and acts in the Ca\(^{2+}\) reabsorption process, which occurs in the distal parts of the nephron. However, there is considerable evidence against the idea that renal calbindin acts exclusively on transcellular reabsorption of Ca\(^{2+}\), which accounts for only a small proportion of Ca\(^{2+}\) reabsorption: (a) renal calbindin remained unchanged or even was reduced in Ca\(^{2+}\)-restricted birds, despite the increases in renal 1,25(OH)\(_2\)D\(_3\) content and the increased reabsorption; but (b) it is markedly or slightly increased, respectively, in P-restricted birds or in those fed high dietary calcium, whether they are fed vitamin D or 1-hydroxylated metabolites of vitamin D; (c) the last two nutritional conditions are associated with a homeostatic need to reduce reabsorption, and with increased urinary calcium. These findings suggest that calbindin may be involved, through a not-yet-understood mechanism, in the removal of plasma calcium in hypercalcemic animals, and that it may also act as a buffer protein that prevents excessive cellular Ca\(^{2+}\), or as an anti-apoptosis factor.

### 3.3. Eggshell Gland

The absence of major effects of vitamin D or estrogens on ESG calbindin, the association of ESG calbindin mRNAs, but not of calbindin with shell formation, and the relationship of ESG calbindin with the mass of Ca\(^{2+}\) transported (eggshell calcium), rather than with the transport capability (as in the intestine), suggests that, similarly to the kidney, the calbindin content, which is related to shell Ca\(^{2+}\) mass, may provide buffering capacity that prevents the accumulation of excessive cellular Ca\(^{2+}\), or that acts as an anti-apoptosis, rather than a transporting factor.

The hypothesis that calbindin, a major component of the transcellular transport of calcium, plays different roles in Ca\(^{2+}\) transport in the intestine, the kidney and the ESG is strengthened by the findings that vitamin D plays different roles in calbindin synthesis by these three transporting organs.

### Acknowledgments

Contribution from the Institute of Animal Science, Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel: No. 537/08. The encouragement of Prof. S. Yahav is acknowledged with appreciation.

### References

- Balmain N. Calbindin-D9k. A vitamin-D-dependent, calcium-binding protein in mineralized tissues. Clinical Orthopaedics


Bar A, Vax E, Hunziker W, Haley O and Striem S. The role of gonadal hormones in gene expression of calbindin (M(r)
28,000) in the laying hen. General and Comparative Endocrinology, 103: 115-122. 1996.

Bar A, Vax E and Striem S. Relationships between calbindin (Mr 28,000) and calcium transport by the eggshell gland. Comparative Biochemistry and Physiology. B: Comparative Biochemistry, 101: 845-848. 1992b.


Corradino RA and Fullmer CS. Stimulation of a Cd-binding protein, and inhibition of the vitamin D-dependent calcium-binding protein, by zinc or cadmium in organ-cultured


Doi O, Takai T, Nakamura T and Tanabe Y. Changes in the pituitary and plasma LH, plasma and follicular progesterone and estradiol, and plasma testosterone and estrone concentrations during the ovulatory cycle of the quail (Coturnix coturnix japonica). General and Comparative Endocrinology, 41: 156-163. 1980.


Hurwitz S, Bar A and Mesher A. Field rickets in turkey pouls:


Lippiello L. Vitamin D-induced calcium binding protein: fluorescent antibody localization in the shell gland (uterus) and kidney and related studies. 1974.


Navickis RJ, Dial OK, Katzenellenbogen BS and Nalbandov AV. Effects of gonadal hormones on calcium-binding pro-


Wu JCY, Smith MW, Turvey A, Keable SJ and Colston KW. Differential regulation of vitamin D receptor and intestinal


