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Functional Involvement of Carbonic Anhydrase in Calcium Transport of the Chick Chorioallantoic Membrane

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Carbonic anhydrase activity was demonstrated in the chick-embryonic chorioallantoic membrane and was correlated with the Ca^{2+}-transport activity of the membrane. It is inhibited by sulphonamides and is expressed in the chorioallantoic membrane in an age-dependent fashion during embryonic development. Ca^{2+} uptake by the chorioallantoic membrane *in vivo* also increases in a similar age-dependent manner. The temporal increase in these activities is coincident with calcium deposition in the embryonic skeleton. Incubation of the chorioallantoic membrane *in ovo* with sulphonamides specifically inhibits both the carbonic anhydrase and the Ca^{2+} uptake activities of the membrane *in vivo*. Enzyme histochemistry revealed that the carbonic anhydrase activity is localized in the Ca^{2+}-transporting ectodermal cells of the chorioallantoic membrane. These results, taken together, indicate that carbonic anhydrase may be functionally important in the Ca^{2+}-transport activity of the chorioallantoic membrane.

The chorioallantoic membrane is an active Ca^{2+}-transporting epithelium of the chick embryo (Terepka *et al.*, 1976). During embryonic skeletal calcification, the chorioallantoic membrane is responsible for mobilizing over 100mg of calcium from the egg shell into the embryonic circulation (Johnston & Comar, 1955; Romanoff, 1967). The Ca^{2+}-transport function of the chorioallantoic membrane is highly developmentally regulated, beginning after the days 12–13 of incubation of the chick embryo, and continuing to increase steadily until hatching (Terepka *et al.*, 1969; Crooks & Simkiss, 1975). Calcium transport is carried out by the ectodermal cells and is highly specific for Ca^{2+} ions (Terepka *et al.*, 1969; Garrison & Terepka, 1972; Terepka *et al.*, 1971). The exact mechanism of Ca^{2+} transport by the chorioallantoic membrane is not yet understood. However, we have identified and isolated from the chorioallantoic membrane a Ca^{2+}-binding protein that appears to be functionally involved in the Ca^{2+}-transport process (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*).

Two other aspects pertinent to the mechanism of Ca^{2+} transport by the chorioallantoic membrane remain unclear. First, how is the calcite ($CaCO_3$) of the egg shell solubilized before Ca^{2+} transport? Several authors (Leeson & Leeson, 1963; Coleman & Terepka, 1972*b*) have suggested that an acid is secreted by the ectodermal cells of the chorioallantoic membrane for this purpose. Second, what processes regulate the metabolic fate of the HCO_3^- liberated from the shell after its solubilization and during active Ca^{2+} transport by the chorioallantoic membrane? A study by Dawes & Simkiss (1971) has indicated that HCO_3^- present in the chick-embryonic blood is partly derived from the solubilization of egg-shell mineral.

The enzymic action of carbonic anhydrase in the reversible hydration of CO_2 and as a regulator of physiological acid–base balance (Maren, 1967; Carter, 1972) suggests that it may be important in these aspects of the Ca^{2+}-transport function of the chorioallantoic membrane. Indeed, carbonic anhydrase has been shown to be involved in active ion transport by various epithelia (see reviews by Maren, 1967; Carter, 1972; Bundy, 1977).

To investigate the possibility of a functional role for carbonic anhydrase in Ca^{2+} transport by the chorioallantoic membrane, we began a comparative study of carbonic anhydrase activity in Ca^{2+}-transporting and non-transporting chorioallantoic membrane isolated from embryos at ages before and after the onset of skeletal calcium deposition. We report here that Ca^{2+}-transporting chorioallantoic membrane exhibits a specific carbonic anhydrase activity that is required in the Ca^{2+}-transport function.

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Materials and Methods

Fertilized white Leghorn eggs were purchased from Shamrock Farms (North Brunswick, NJ, U.S.A.) or SPAFAS Co. (Norwich, CT, U.S.A.) and incubated at 37.5°C in a humidified egg incubator for the desired periods of time. The chorioallantoic membrane was dissected out from the embryos and extracts of the membrane were prepared as previously described (Tuan & Scott, 1977) in 0.02M-Tris/HCl buffer, pH8.0.

Carbonic anhydrase was measured at 0°C by the electrometric method of Wilbur & Anderson (1948) with CO_2 as the substrate. Ca^{2+}-binding activity was assayed by the Chelex 100 ion-exchange method (Tuan & Scott, 1977). Determination of haemoglobin was carried out by the cyanomethaemoglobin method (Cannan, 1958) with bovine haemoglobin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard.

Ca^{2+} uptake by the chorioallantoic membrane *in vivo* was measured by a modification of the method of Crooks & Simkiss (1975) and Crooks *et al.* (1976) with $^{45}CaCl_2$ (New England Nuclear, Boston, MA, U.S.A.; 1–2μCi/μmol of ^{40}Ca) as at racer in the uptake buffer (1.2mM-$MgSO_4$, 25.0mM-$NaHCO_3$, 0.11M-NaCl, 6.0mM-KCl, 1.0mM-Na_2HPO_4, 4.4mM-glucose, 1.0mM-$CaCl_2$, pH7.4). Embryos of the appropriate ages were removed from the incubator and kept at room temperature for 2–3h until they had equilibrated to approx. 25°C. The polar region of the chorioallantoic membrane with the overlying inner shell membrane, located under the air space, was exposed by removing a piece of the egg shell (approx. 2cm^2) from the blunt end of the egg. An annulus 5mm high was constructed from Tygon tubing of internal diameter 7.94mm to define an area of 49.5mm^2. The Ca^{2+}-uptake chamber was completed by greasing one surface of the annulus with silicone grease and pressing it lightly on to the central area of the membrane surface. A portion (200μl) of the ^{45}Ca-containing buffer was placed in the annulus and the egg was gently rotated to wet the whole area of the membrane enclosed by the annulus. At specific time intervals, the buffer was removed, and the membrane surface blotted dry. The annulus was detached and the membrane directly under it was removed by dissection. The membrane samples were immediately washed with two or three changes of cold physiological saline (0.9% NaCl), placed into glass scintillation vials, and digested with 1ml of Protosol (New England Nuclear) according to the manufacturer's instructions. The digested samples were then mixed with 10ml of Aquasol (New England Nuclear), and radioactivity was determined by liquid-scintillation counting. Ca^{2+}-uptake activities were expressed as nmol of Ca^{2+}/min per cm^2.

Carbonic anhydrase was demonstrated histochemically by the method of Hansson (1968) modified as described by Lönnerholm (1974). Samples of chorioallantoic membrane (0.5mm^2 strips) were fixed in glutaraldehyde, frozen-embedded in Tissue-Tek (Tek-Products, Naperville, IL, U.S.A.) and cryosectioned at 5–6μm thickness. The chorioallantoic membrane sections were picked up on Millipore filters (0.45μm) and stained for carbonic anhydrase activity by flotation on a buffer solution containing $CoSO_4$, followed by a blackening of the reaction product with $(NH_4)_2S$. The cell fractions used for carbonic anhydrase histochemistry were dissociated from the chorioallantoic-membrane by an enzymic procedure described elsewhere (Tuan *et al.*, 1978*b*). The chorioallantoic membrane cells were smeared on to Millipore filters (0.45μm), air-dried, fixed in 10% formaldehyde in 95% ethanol for 5min, and stained as described for tissue sections. The Millipore filters with the chorioallantoic-membrane sections or cell smears were then dehydrated and cleared in a series of ethanol, propan-1-ol and xylene washes, mounted on a glass slide, and immediately observed under the optical microscope.

Inhibitors of carbonic anhydrase used in this study include: acetazolamide (Sigma), sulphanilamide (Merck, Rahway, NJ, U.S.A.) and toluene-*p*-sulphonamide (Pfaltz and Bauer, Stamford, CT, U.S.A.). All other chemicals used were of reagent grade.

Results and Discussion

The carbonic anhydrase activity per milligram of protein in extracts of chorioallantoic membrane was measured as a function of the embryonic age. As shown in Fig. 1, enzyme activity is undetectable at early stages of development before incubation day 11. Between incubation days 12 and 14, however, the activity increases and rises rapidly thereafter. The maximal carbonic anhydrase activity in the chorioallantoic membrane occurs around day 19 of incubation, after which, before hatching on day 21, the activity declines slightly. The increased carbonic anhydrase activity in the chorioallantoic membrane during later stages of embryonic development appears to be largely of tissue origin, since the contamination by erythrocytic carbonic anhydrase activity is relatively insignificant (Fig. 1).

The carbonic anhydrase activity of the chorioallantoic membrane is inhibited by several specific sulphonamide-type carbonic anhydrase inhibitors (Maren, 1967), including acetazolamide, sulphanilamide and toluene-*p*-sulphonamide (Fig. 2). The I_{50}

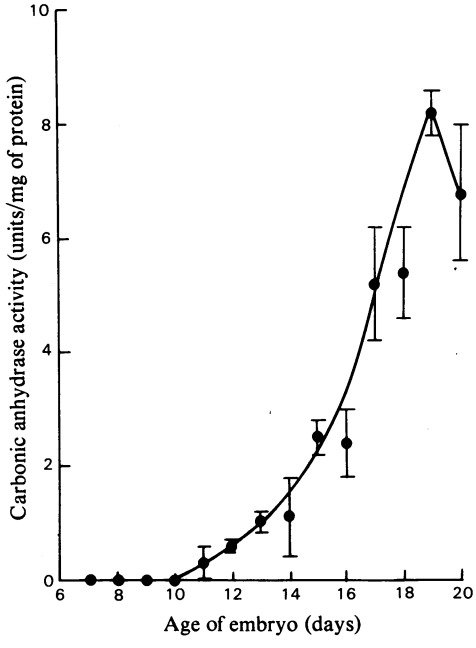


Fig. 1. *Age profile of the specific carbonic anhydrase activity of chorioallantoic-membrane extracts*

The enzyme activity was determined by the method of Wilbur & Anderson (1948), where the time required for a saturated CO_2 solution to lower the pH of 0.02M-Tris/HCl buffer from 8.3 to 6.3 at 0°C was determined. The time without enzyme was recorded as t_0 and, with enzyme, t. Units of carbonic anhydrase activity are calculated as $2\times(t_0-t)/t$. The specific carbonic anhydrase activities (activity/mg of protein) are the means ± S.E.M. of at least three determinations on soluble extracts of pooled membranes from five or more embryos. The carbonic anhydrase activity in the chorioallantoic-membrane extract attributable to erythrocytes was determined as described by Clark (1951) by first measuring the enzyme activity in the embryonic blood and then determining the concentration of haemoglobin in both the blood and the chorioallantoic-membrane extract. In all cases, the blood-derived carbonic anhydrase contamination calculated from the activity/haemoglobin ratios was less than 2% of the total activity.

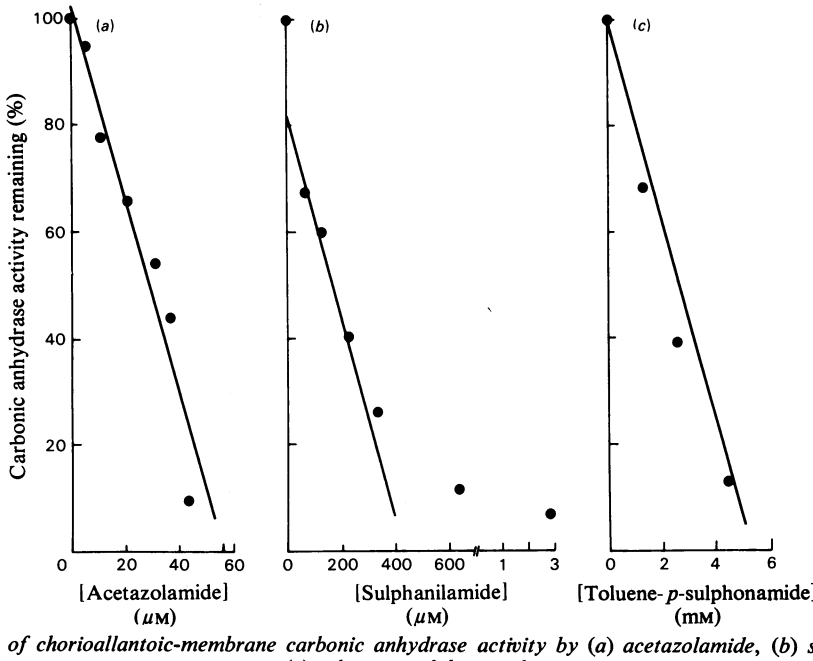


Fig. 2. *Inhibition of chorioallantoic-membrane carbonic anhydrase activity by (a) acetazolamide, (b) sulphanilamide and (c) toluene-p-sulphonamide*

The chorioallantoic-membrane extracts were prepared as described in the Materials and Methods section from 19-day embryos. Carbonic anhydrase activity was determined as described in the Materials and Methods section. The sulphonamide-type inhibitors were added to the reaction mixture at the indicated final concentrations immediately before determination of enzyme activity. Controls containing the inhibitors alone exhibited no carbonic anhydrase activity.

values (concentration of inhibitors required to inhibit 50% of the enzyme activity in chorioallantoic membrane extracts prepared from 19-day embryos) are: acetazolamide, 28.8 μM; sulphanilamide, 193 μM; toluene-*p*-sulphonamide, 2.65 mM. The relative potency of these compounds to inhibit the chorioallantoic-membrane carbonic anhydrase activity is thus comparable with that obtained for the carbonic anhydrase activities from various other tissues (Maren, 1967; Carter, 1972). Together, these results therefore indicate that a specific carbonic anhydrase activity is present in the chorioallantoic membrane of the chick embryo, and that it is expressed in an age-dependent manner during development.

As pointed out in the introduction, the capacity of the chorioallantoic membrane to mobilize Ca^{2+} from the egg shell is also a developmentally regulated function (Johnston & Comar, 1955; Romanoff, 1967; Terepka *et al.*, 1969, 1976). On comparison, it is evident that both the chorioallantoic-membrane Ca^{2+}-transport activity measured *in vitro* (Terepka *et al.*, 1969, 1971) and the accumulation of Ca^{2+} by the embryo (Johnston & Comar, 1955; Romanoff, 1967) exhibit age–activity profiles similar to that of the chorioallantoic-membrane carbonic anhydrase activity (Fig. 1). The temporal correlation between these activities and the carbonic anhydrase activity of the chorioallantoic membrane strongly suggests that the latter may be functionally important in the Ca^{2+}-transport process of the chorioallantoic membrane.

To characterize the relationship between these activities further, it is necessary first to confirm and establish the developmental dependence of the chorioallantoic-membrane Ca^{2+}-transport function. Previously, Terepka *et al.* (1969, 1971) have shown, by means of an Ussing chamber-type apparatus, that the rate of Ca^{2+} transport by the chorioallantoic membrane measured *in vitro* exhibits an age-dependent increase subsequent to incubation day 14. Their findings, together with the observable progressive increase in skeletal calcification of the embryo during development (Romanoff, 1967), strongly suggest that, *in vivo*, the Ca^{2+}-transport function of the chorioallantoic membrane should also increase in capacity as the embryo matures. This supposition was tested here by means of a Ca^{2+}-transport system *in vivo* (Crooks & Simkiss, 1975; Crooks *et al.*, 1976). Initial kinetic studies showed that a linear rate of Ca^{2+} uptake *in vivo* by the chorioallantoic membrane was maintained for up to 20 min of incubation. These observations confirm the earlier studies by Crooks & Simkiss (1975) and Crooks *et al.* (1976) and demonstrate that these measurements are valid representations of the rates of Ca^{2+} transport *in vivo* by the chorioallantoic membrane. With this technique, the age profile of the Ca^{2+}-transport activity of the chorioallantoic membrane *in vivo* was determined and is shown in Fig. 3. The transport rate is low before incubation days 12–14, rises rapidly thereafter, reaches a maximum around incubation days 19 and 20, and declines slightly before hatching. On comparison, the overall temporal similarity between the age profiles of carbonic anhydrase activity and of Ca^{2+}-transport activity of the chorioallantoic membrane is consistent with a functional relationship between these two parameters.

The functional role of carbonic anhydrase in the Ca^{2+}-transport activity of the chorioallantoic membrane was next investigated by employing the enzyme inhibitors sulphanilamide and acetazolamide in the transport system *in vivo*. As shown in Fig. 4, the incorporation of acetazolamide or sulphanilamide into the uptake buffer resulted in a substantial decrease in the Ca^{2+}-uptake activity of the chorioallantoic membrane. Some 50% of the transport activity was observed in the presence of 30 μM-acetazolamide or 0.5 mM-sulphanilamide. These concentrations are comparable with the I_{50} values (Fig. 2) for the inhibition of carbonic anhydrase activity by the respective inhibitor in cell-free chorioallantoic-membrane extracts. In addition, under these incubation conditions, the carbonic anhydrase activity of the chorioallantoic membrane appeared to be readily accessible to, and inhibited by, these sulphonamide compounds. Our results (Fig. 4) show that the carbonic anhydrase activity

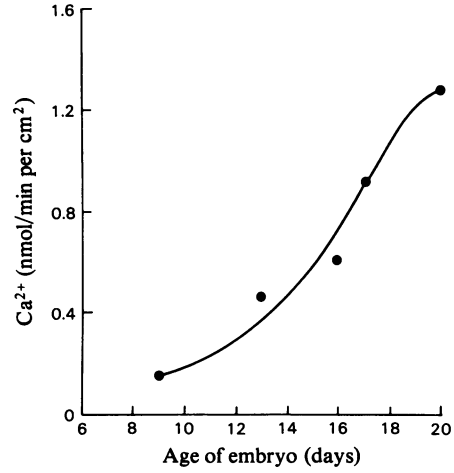


Fig. 3. *Age profile of Ca^{2+}-uptake activity in vivo of the chorioallantoic membrane*

Rates of Ca^{2+} uptake (nmol of Ca^{2+}/min per cm^2) by the chorioallantoic membrane were determined as described in the Materials and Methods section from the initial rates measured at 15 min and represent the average values from three determinations, four to eight embryos being used each time.

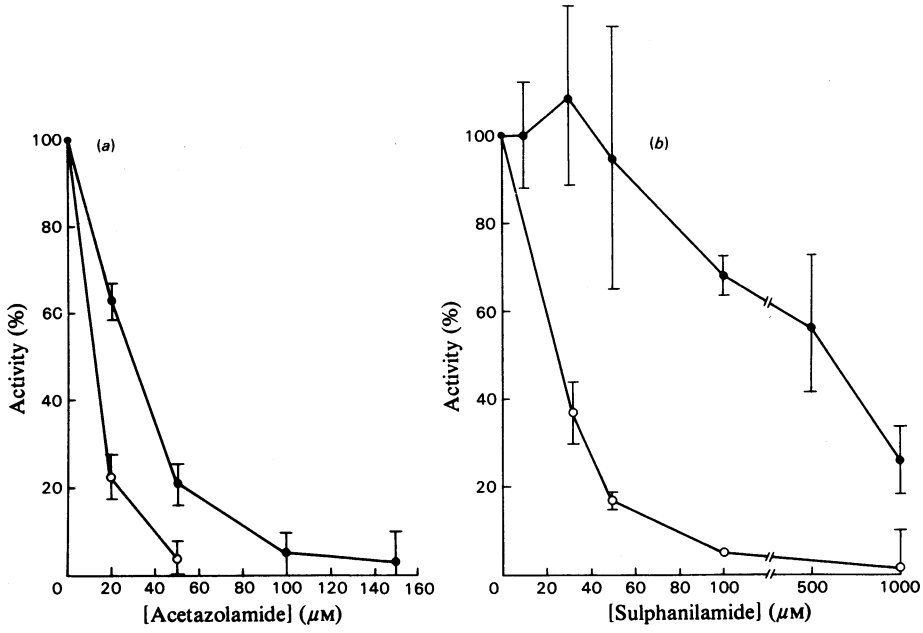


Fig. 4. *Inhibition of Ca^{2+} uptake and carbonic anhydrase activities of the chorioallantoic membrane by administration in vivo of (a) acetazolamide and (b) sulphanilamide*

The inhibitors were incorporated at the indicated concentrations into the Ca^{2+}-uptake buffer and the chorioallantoic membrane of 17-day embryos were incubated for 15min with the inhibitor-containing buffer, with or without ^{45}Ca, by means of the uptake chamber (see the Materials and Methods section). The underlying chorioallantoic membrane was then dissected out and washed. Ca^{2+}-uptake activities (nmol of Ca^{2+}/min per cm^2) were measured as described in the Materials and Methods section and carbonic anhydrase activities (units/mg of protein) were determined in extracts of the chorioallantoic membrane (see the Materials and Methods section). The respective activities (Ca^{2+} uptake, ●; carbonic anhydrase, ○) are expressed as a percentage of that incubated with buffer alone. The values represented are the means ± S.E.M. of two to three determinations, six to eight embryos being used for each.

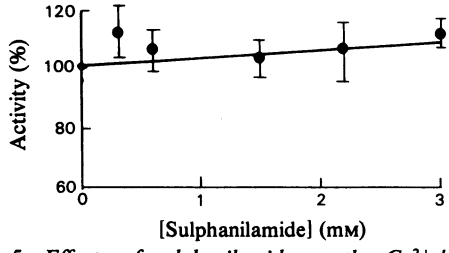


Fig. 5. *Effects of sulphanilamide on the Ca^{2+}-binding activity of chorioallantoic-membrane extract from 19-day embryos*

The chorioallantoic-membrane extract was prepared and Ca^{2+}-binding activities were determined as described previously (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*). Sulphanilamide was incorporated into the extract at the indicated concentrations immediately before the Ca^{2+}-binding assay. The activities in the presence of sulphanilamide were expressed as a percentage of that in the absence of the inhibitor. The values represented are the means and S.E.M. of four determinations. Controls containing sulphanilamide alone exhibited no Ca^{2+}-binding activity.

in the extracts prepared from the segment of chorioallantoic membrane directly forming the uptake chamber is specifically inhibited. In comparison, another transport-related function, the Ca^{2+}-binding activity of the chorioallantoic membrane, is not affected (Fig. 5). Furthermore the relative potency of acetazolamide and sulphanilamide to inhibit carbonic anhydrase activity and Ca^{2+} transport *in vivo* is comparable with that obtained for enzyme inhibition (Fig. 2) in cell-free extracts of the chorioallantoic membrane. Taken together, these results provide further evidence for a functional involvement of carbonic anhydrase activity in the Ca^{2+}-transport activity of the chorioallantoic membrane.

Finally, we studied the properties of the chorioallantoic-membrane carbonic anhydrase activity by means of enzyme histochemistry. The enzyme activity was histochemically demonstrated in the chorioallantoic membrane with the $CoSO_4$-staining method (see the Materials and Methods section). Plate 1(*a*) shows the staining pattern obtained with frozen sections of chorioallantoic membrane from

19-day embryos. In addition to the expected association with erythrocytes (Maren, 1967; Carter, 1972; Bundy, 1977) found in the blood vessels, distinct dark cobaltous deposits were exclusively localized along the ectodermal layer of the chorioallantoic membrane, which has been previously shown to contain the Ca^{2+}-transporting cells of the chorioallantoic membrane (Coleman & Terepka, 1972*a*; Armbrecht *et al.*, 1976). At a higher magnification (Plate 1*b*), the ectodermal stains appear to be cortically distributed in the columnar epithelial cells of the ectoderm and, in many cases, outlining these cells. Similar staining patterns were observed in cells dissociated from the chorioallantoic membrane (Plate 1*c*). These cells have been previously shown to contain also the chorioallantoic-membrane Ca^{2+}-binding protein (Tuan *et al.*, 1978*b*). Control experiments performed to test the specificity of the staining in the 19-day chorioallantoic membrane included: incorporation of acetazolamide or sulphanilamide in the incubation medium, and omission of $CoSO_4$ or $(NH_4)_2S$ from the incubation mixture. All controls failed to reveal any positive staining at either the erythrocytic or ectodermal sites of the 19-day chorioallantoic membrane. Furthermore staining was also absent from 19-day chorioallantoic membrane that was treated with sulphanilamide (or acetazolamide) during Ca^{2+} transport *in vivo*. Correlation between carbonic anhydrase activity and Ca^{2+}-transport activity was further provided from the observation that the ectoderm of 11-day-old non-transporting chorioallantoic membrane revealed no carbonic anhydrase-positive staining. These results therefore indicate that functional Ca^{2+}-transport activity in the chorioallantoic membrane is always accompanied by carbonic anhydrase activity.

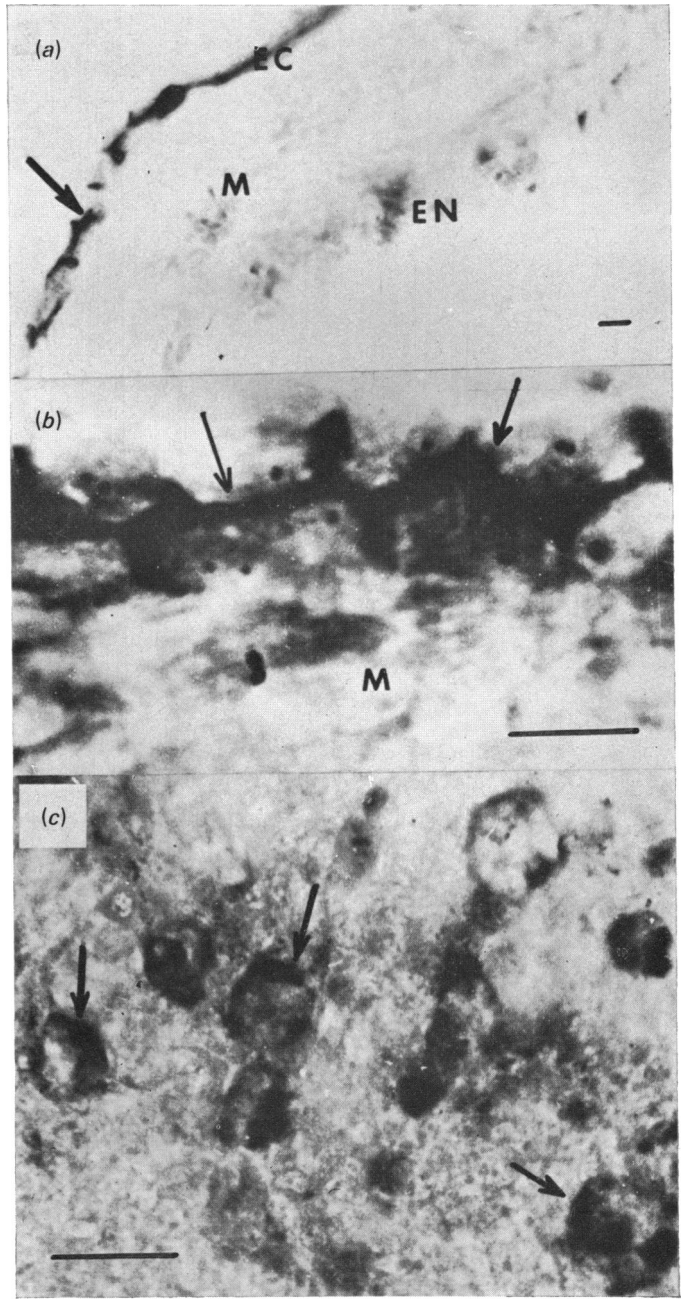
Overall, the results presented here reveal that a specific non-erythrocytic carbonic anhydrase activity is expressed in the ectodermal cells of the chorioallantoic membrane as a function of embryonic development, and that the enzyme activity is absent from the chorioallantoic membrane under non-transporting conditions, such as during early stages of development or in the presence of sulphonamides.

From the present study, three lines of functional correlation between the Ca^{2+}-transport activity and the carbonic anhydrase activity of the chorioallantoic membrane are apparent: (*a*) the two activities are expressed concomitantly during embryonic development; (*b*) both activities are inhibited by specific sulphonamide-type carbonic anhydrase inhibitors; (*c*) carbonic anhydrase is associated with the Ca^{2+}-transporting ectodermal cells of the chorioallantoic membrane.

The developmentally programmed process of skeletal calcification in the chick embryo requires a high degree of regulation of the mechanisms involved in the mobilization of calcium by the chorioallantoic membrane from the egg shell. One of the mechanisms appears to be the expression of a specific Ca^{2+}-binding protein (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*). In this report we have presented evidence strongly indicating that the expression of carbonic anhydrase activity is also important in the Ca^{2+}-transport function of the chorioallantoic membrane. Though the two activities are expressed concomitantly in the chorioallantoic membrane during development (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*) and are histochemically localized in the same population of cells (Tuan *et al.*, 1978*b*), it appears unlikely that they are derived from the same molecular species. Sulphonamides strongly inhibit the carbonic anhydrase activity, but not the Ca^{2+}-binding activity in chorioallantoic-membrane extracts. Moreover, the physicochemical properties of the Ca^{2+}-binding protein (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*) differ significantly from the known properties of carbonic anhydrase isolated from various species (Maren, 1967; Carter, 1972).

The expression of carbonic anhydrase during embryonic development has been studied by many investigators. Clark (1951) showed that carbonic anhydrase is expressed with characteristic age-specific patterns in various embryonic tissues and suggested that the enzyme activity is correlated with the functional maturity of the tissues. Perturbation of carbonic anhydrase activity in the developing chick embryo *in ovo* by the administration of sulphonamides has been reported (Landauer & Clark, 1964; Landauer & Wakasugi, 1967) to produce gross malformations, particularly in the embryonic skeleton. These anatomical abnormalities include micromelia, deformity of the upper beak and bending of the tibiotarsus shaft. Owing to the ubiquitous nature of carbonic anhydrase in the physiological buffering system of many tissues (Maren, 1967; Carter, 1972; Bundy, 1977), these observations do not, by themselves, directly indicate the functional importance of the enzyme in the uptake of Ca^{2+} by the embryonic chorioallantoic membrane. The observation of skeletal malformations is nevertheless consistent with a perturbation in the calcium metabolism of the embryo as a result of inhibition of carbonic anhydrase activity.

Though our present study strongly indicates the functional involvement of carbonic anhydrase in the Ca^{2+}-transport process of the chorioallantoic membrane, the exact role of the enzyme remains unclear. Our determinations showing the temporal correlation between the onset of carbonic anhydrase activity in the chorioallantoic membrane and accumulation of Ca^{2+} by the embryo are inherently insufficiently refined to resolve the possible causal relationship between the two parameters. It is conceivable, as suggested in the introduction, that the enzyme is



EXPLANATION OF PLATE 1

Histochemical localization of carbonic anhydrase activity in the chorioallantoic membrane of a 19-day chick embryo (*a*) Cryosection, 6μm thick. (*b*) Cryosection, 6μm thick. (*c*) Dissociated cells, prepared according to procedure of Tuan *et al.* (1978*b*). All samples were placed on Millipore filters, 0.45μm thick, and stained by the $CoSO_4$ method as described in the Materials and Methods section. Arrows indicate carbonic anhydrase-positive staining. The general granular background was due to the presence of the Millipore filter used as a support during staining which was mounted with the samples on to the glass slides. Abbreviations: EC, ectoderm; M, mesoderm; EN, endoderm. Bars denote 10μm.

involved in either the solubilization of the shell calcite reserve through localized acidification (Coleman & Terepka, 1972*b*) or the metabolic scavenging of the HCO_3^- released from the shell, or both. The rapid action of carbonic anhydrase inhibitors such as acetazolamide and sulphanilamide in inhibiting Ca^{2+} uptake by the chorioallantoic membrane *in vivo* appears to favour the involvement of the enzyme in the relatively faster HCO_3^--scavenging mechanism. It needs to be pointed out that the inhibitory effects of acetazolamide and sulphanilamide are slightly dissimilar in that the inhibition of Ca^{2+} uptake and carbonic anhydrase activity with the former parallel each other, whereas there is a less precise correlation of the inhibition of these two activities with the latter. Whether this is due to dissimilarities in the mode of inhibition, enzyme-binding affinity or other properties of the two sulphonamides remains to be resolved. Interestingly, since complete inhibition of carbonic anhydrase activity by sulphonamides does not result in total inactivation of Ca^{2+} uptake by the chorioallantoic membrane (Fig. 4), it is unlikely that the enzyme is solely responsible for the transport process. Additional mechanisms, involving components such as the Ca^{2+}-binding protein (Tuan & Scott, 1977; Tuan *et al.*, 1978*a*), are necessarily operative in the Ca^{2+}-transport function of the chorioallantoic membrane. In other systems, such as the kidney (McKinley & Whitney, 1976) and the pituitary (Kimura & MacLeod, 1975), carbonic anhydrase appears to be localized and functional both at the plasma membrane and in the cytosol, possibly in the form of different isoenzymes (Carter, 1972). Whether similar subcellular distribution and functional mechanisms of carbonic anhydrase are present in the chorioallantoic membrane remains to be investigated.

Carbonic anhydrase has been directly implicated in a highly active and specific Ca^{2+}-transporting tissue, the avian shell gland, by Pearson and coworkers (Pearson & Goldner, 1973, 1974; Pearson *et al.*, 1977). These investigators demonstrated that carbonic anhydrase activity in the shell gland is elevated during the egg-laying cycle of the hen and that Ca^{2+} transport by the tissue measured *in vitro* is strongly inhibited by sulphonamides. It is thus noteworthy that our present study suggests that the solubilization and mobilization of calcium from the egg shell, the product of the uterine shell gland, may be carried out by the embryonic placenta-like chorioallantoic membrane via a carbonic anhydrase-related mechanism.

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