oxidase and a variety of acid hydrolases were distributed in the gradients at a mean density of approximately 1.23, which correlates well with the data of others (*10*). Most of the acid and alkaline phosphatase activities, measured with *p*-nitrophenyl phosphate as substrate (*11*), were considerably closer to the top of the gradients. This seeming discrepancy in distribution of acid phosphatase and (other) lysosomal hydrolases has been noted (*8*) with rabbit heterophile leukocytes and related to the presence of two acid phosphatases in these cells. In man, as in the rabbit, alkaline phosphatase of the leukocyte does not appear to be compartmentalized with the acid hydrolases and peroxidase.

In cystinotic leukocytes a large percentage of cystine migrated in the gradients with an average bouyant density of 1.23, along with the dense lysosomal particles. Cystinotic cells show this characteristic cystine peak after labeling with brief exposure to cysteine-^{35}S and also by the quantitative colorimetric technique, which is independent of tracer methodology and hence not influenced by rates of incorporation of exogenously supplied cysteine into intracellular cystine pools (Figs. 1 and 2). This cystine peak deep in the gradient was not seen in preparations of normal cells. In preparations of both normal and cystinotic cells, some cystine was always seen at the top of the gradients; this presumably represents noncompartmentalized cystine which had in part preexisted in these cells as the normal constituent cysteine, and possibly some cystine which had leaked out of intracellular organelles during preparation.

The small numbers of leukocytes available precluded identification of the mitochondrial band by enzyme assay in our gradients. However, mitochondria of human neutrophiles have a bouyant density of approximately 1.17 (*10*), a value that corresponds to that of tube 10 in our gradients where only minute amounts of cystine were detected.

The specific gravity of cystine crystals is approximately 1.73 (*12*). The fact that the cystine peak characteristic of cystinotic leukocyte gradients is found at the very much lower specific gravity of 1.23 indicates that the cystine occupying the lysosomes in leukocytes is probably not in the crystalline form in which it has been visualized in cystinotic reticuloendothelial cells from lymph nodes and in conjunctival macrophages. Cystine, predominantly in noncrystalline form, is apparently compartmentalized within a dense class of lysosomes in cystinotic leukocytes.

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References and Notes

1. H. Bickel, H. S. Barr, R. Astley, A. A. Douglas, H. Harris, E. M. Hickmans, W. C. Smallwood, J. M. Smellie, C. G. Teall, *Acta Paediat.* **42**, Suppl. 90, 7 (1952); J. E. Seegmiller, T. Friedmann, H. E. Harrison, V. Wong, J. A. Schneider, *Ann. Intern. Med.* **68**, 883 (1968).
2. J. A. Schneider, K. H. Bradley, J. E. Seegmiller, *Science* **157**, 1321 (1967); J. A. Schneider, F. M. Rosenbloom, K. H. Bradley, J. E. Seegmiller, *Biochem. Biophys. Res. Commun.* **29**, 527 (1967).
3. A. D. Patrick and B. D. Lake, *J. Clin. Pathol.* **21**, 571 (1968); V. Wong, T. Kuwabara, R. Brubaker, J. D. Schulman, J. E. Seegmiller, in preparation.
4. R. Morecki, L. Paunier, J. R. Hamilton, *Arch. Pathol.* **86**, 297 (1968).
5. J. D. Schulman, V. Wong, T. Kuwabara, K. H. Bradley, J. E. Seegmiller, *Arch. Intern. Med.*, in press.
6. J. A. Schneider, K. H. Bradley, J. E. Seegmiller, *Pediat. Res.* **2**, 441 (1968).
7. A. C. Allison and M. R. Young, *Life Sci.* **3**, 1407 (1964).
8. M. Baggiolini, J. G. Hirsch, C. de Duve, *J. Cell Biol.* **40**, 529 (1969).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951); W. E. Bowers, J. T. Finkenstaedt, C. de Duve, *J. Cell Biol.* **32**, 321 (1967).
10. J. Schultz, R. Corlin, F. Oddi, K. Kaminker, W. Jones, *Arch. Biochem. Biophys.* **111**, 73 (1965).
11. *Sigma Technical Bulletin No. 104* (1963).
12. J. D. Schulman, unpublished data.

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Intestinal Calcium Absorption: Nature of Defect in Chronic Renal Disease

Abstract. *When compared to that of normal animals, calcium-binding protein activity of duodenal mucosa obtained from uremic rats was decreased. There was no change in this activity after vitamin D_3 therapy. In contrast, prior treatment with 25-hydroxycholecalciferol resulted in increased transport of calcium-45 and calcium-binding protein activity in the intestine.*

The intestinal absorption of calcium is characteristically depressed in patients with chronic renal insufficiency (*1*) and in rats with experimentally induced chronic uremia (*2*). We have presented evidence for an abnormal metabolism of vitamin D_3 in subjects with chronic uremia and a decrease in concentration of a biologically active vitamin D_3 metabolite in the plasma (*3*). This metabolite, which is normally more potent than vitamin D_3 in effecting the intestinal transport of calcium, has been subsequently identified by De Luca and co-workers as 25-hydroxycholecalciferol (*4*). In our earlier studies we suggested that the derangements of vitamin D_3 metabolism noted in uremic subjects could account, at least in part, for the intestinal malaborption of calcium of chronic renal disease and the reported "resistance" of renal osteodystrophy to vitamin D therapy (*3*). Since there is mounting evidence that some of the effects of vitamin D or metabolites thereof on intestinal calcium transport may be mediated by the induction of a specific calcium-binding protein (Ca BP) (*5*), the possibility existed that, as a consequence of abnormal vitamin D metabolism, the activity of Ca BP was depressed in the chronic uremic state.

We now report that the concentration of Ca BP is decreased in the intestinal mucosa of rats with experimentally induced chronic uremia and defective intestinal calcium transport. Whereas concomitant increments in calcium transport and amounts of intestinal duodenal Ca BP were observed in normal rats after vitamin D_3 therapy, vitamin D_3 was ineffectual in reversing both the abnormal calcium transport and low concentrations of Ca BP in the mucosa of uremic rats. In contrast, 25-hydroxycholecalciferol was extremely effective in reversing the abnormalities in calcium transport and Ca BP activity.

The chronic uremic state was experimentally induced in male Sprague-Dawley rats (100 g) by unilateral (right) nephrectomy and ligation of most of the primary and secondary divisions of the main left renal artery. Sixty days after nephrectomy and segmental infarction of the contralateral kidney, when urea nitrogen in the blood was 70 mg per 100 ml or greater, the animals were placed on a low calcium diet for 4 days and then killed by decapitation. At death, the uremic animals were randomly distributed into one of two groups. In one group a

3- to 4-cm segment of proximal duodenum was excised from each animal and everted, and ^{45}Ca transport was measured in vitro by the everted gut sac techniques (*6*). In the second group, the duodenum was immediately excised, cooled to 4°C, slit open, rinsed with cold 0.12*M* NaCl and blotted dry. Mucosal tissue was scraped from the underlying muscle layer with a glass slide, and the pooled harvest (from 14 uremic animals), was homogenized in tris buffer (20 percent, weight/volume) with a Potter-Elvehjem homogenizer and a Teflon pestle. The crude homogenate was spun at 38,000*g* for 20 minutes. The supernatant was removed and heated to 60°C for 10 minutes, cooled, and centrifuged again at 38,000*g* for 20 minutes. The calcium-binding activity of a portion of the heat-treated supernatant was determined by the competitive Chelex-100 resin binding assay (*7*). The protein content of this portion was measured by the Lowry method (*8*). The remainder of the heat-treated crude mucosal supernatant was concentrated on a Diaflo membrane filter and chromatographed on Sephadex G-100 at 5°C with tris buffer as the eluting agent (*9*); 200 fractions containing 2.5 ml were collected. Alternate Sephadex fractions were analyzed for protein and calcium-binding activity. Pair-fed sham-operated control animals with ages similar to the uremic group were also placed on a diet low in calcium for 4 days and then killed, and both ^{45}Ca transport and Ca BP activity of crude and Sephadex-fractionated supernatants from duodenal homogenate were measured as outlined above.

The effect of orally administered vitamin D_3 and 25-hydroxycholecalciferol on the ability of the isolated gut sacs to transport ^{45}Ca and the concentration of Ca BP in the mucosa were also tested. Uremic and control animals each received 500 units of either vitamin D_3 or 25-hydroxycholecalciferol in cottonseed oil orally 12 hours before they were killed. At that time ^{45}Ca transport and Ca BP studies were performed as outlined for the untreated normal control and uremic animals.

In the uremic animals ^{45}Ca transport of isolated duodenal gut sacs was depressed (Table 1). The average ratio of the ^{45}Ca concentration in the serosal side to that in the mucosal side in rats with experimentally induced chronic uremia was significantly lower than in the controls. These findings are consistent with the observations of Kessner and Epstein, who reported that chronic renal insufficiency in rats decreases the abiltiy of isolated duodenal sacs to transport calcium against a chemical gradient (*2*). Whereas a significant increase ($P < .01$) in ^{45}Ca transport was noted in normal animals after vitamin D_3 therapy, vitamin D_3 failed to reverse the duodenal ^{45}Ca transport of uremic animals. In contrast, prior treatment with 25-hydroxycholecalciferol resulted in a twofold increment in ^{45}Ca transport of everted duodenal sacs obtained from rats with chronic uremia.

When compared to that of normal control animals, mucosa from uremic rats (Table 2) showed a significant ($P < .001$) decrease in Ca BP activity. Moreover, no change in calcium-binding protein was observed in the uremic animals treated with vitamin D_3, whereas the binding activity of the supernatants from the duodenum homogenates of uremic rats treated with 25-hydroxycholecalciferol was 2.9 times greater than that of the nontreated

Table 1. Calcium transport in everted intestinal sacs of normal and uremic rats: effects of vitamin D_3 and 25-hydroxycholecalciferol. Normal and uremic rats were given orally either 500 I.U. of vitamin D_3 or 500 I.U. of 25-hydroxycholecalciferol in 0.1 ml of cottonseed oil 12 hours before they were killed. The controls received the oil alone. Calcium transport, expressed as a ratio of ^{45}Ca on the serosal side to that on the mucosal side, was measured as described (*7*). The values are expressed as mean ± S.E.

Group	Rats (No.)	Dosage (unit)	^{45}Ca(S/M)	*P*
Normal control	10		2.5 ± 0.3	
Normal + vitamin D_3	10	500	3.8 ± 0.2	< .01*
Normal + 25-OH-cholecalciferol	9	500	4.8 ± 0.1	< .01*
Uremic control	10		1.2 ± 0.3	
Uremic + vitamin D_3	11	500	1.5 ± 0.2	> .10†
Uremic + 25-OH-cholecalciferol	10	500	2.5 ± 0.2	< .01†

* As compared to normal control. † As compared to uremic control.

Table 2. Effect of vitamin D_3 and 25-hydroxycholecalciferol on the calcium-binding activity in duodenum of normal and uremic rats. Normal and uremic rats were given orally either 500 I.U. of vitamin D_3 or 500 I.U. of 25-hydroxycholecalciferol in 0.1 ml cottonseed oil; the controls received the oil vehicle alone. Mucosal scrapings of 14 rats were harvested for each determination. The results represent the mean ± standard error of the mean for each group.

Group	Tests (No.)	^{45}Ca in supernatant (%/ml)	Protein (mg/ml)	Specific activity (%^{45}Ca/mg protein)
Normal control	9	10.6 ± 0.4	1.58 ± 0.06	6.7 ± 0.2
Normal + vitamin D_3	7	11.9 ± 0.5	1.48 ± 0.07	7.9 ± 0.2
Normal + 25-OH-cholecalciferol	8	9.6 ± 0.2	0.99 ± 0.02	9.6 ± 0.3
Uremic control	7	3.9 ± 0.6	1.37 ± 0.08	3.0 ± 0.3
Uremic + vitamin D_3	7	4.1 ± 0.3	1.40 ± 0.08	3.0 ± 0.3
Uremic + 25-OH-cholecalciferol	9	11.0 ± 0.4	1.29 ± 0.06	8.6 ± 0.3

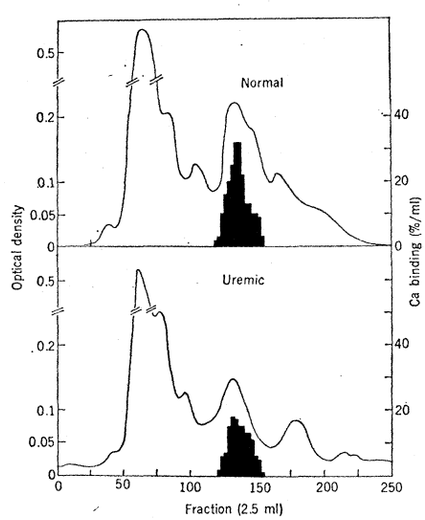

Fig. 1. Distribution of proteins and calcium-binding activity in supernatant fractions of heat-treated supernatants from homogenized mucosa of normal (upper panel) and uremic (lower panel) rats, after Diaflo and dextran-gel filtration (Sephadex G-100). Elution was performed with *p*H 7.4 tris buffer at 5°C, and 2.5-ml fractions were collected. Protein was measured densitometrically in each tube. Portions of alternate fractions were also assayed for protein (*8*) and calcium-binding (black histogram) activity (*7*).

uremic control group. Further purification of the Ca BP with Sephadex G-100 chromatography revealed that the Ca BP activity in both normal and uremic rats was associated with a specific protein fraction and therefore was not a general property of all the proteins contained in the crude heat-treated supernatant (Fig. 1). This isolation procedure resulted in a protein fraction (tubes 140 to 155) which contained all the Ca BP activity in the mucosa homogenates of untreated animals as well as from those treated with vitamin D_3 and 25-hydroxycholecalciferol.

Like that of the crude heat-treated supernatant from mucosa homogenates of uremic animals (Table 2), the binding activity of material isolated by Sephadex chromatography rose minimally after vitamin D_3 treatment but more than twofold after administration of the 25-hydroxycholecalciferol vitamin D_3 metabolite. Because 10 to 16 hours may be required before the defective intestinal calcium absorption is measurably restored to normal by oral vitamin D_3 therapy (*10*), calcium-binding activity of duodenal mucosa was also measured in uremic rats 24 and 48 hours after 500 units of vitamin were administered. In each instance, no significant increment in Ca BP was demonstrable when compared to the Ca BP activity of uremic rat mucosa obtained 12 hours after vitamin D_3 administration.

So far, vitamin D dependent Ca BP has been detected in the intestinal mucosa of the rat, dog, and monkey (*11*). The nature of the interaction of vitamin D with the mucosal cell that leads to the formation of the calcium-binding protein is still in doubt. Studies by Zull, Czarnowska-Misztal, and De Luca (*12*) and Norman (*13*) suggest that this interaction represents either a direct effect of 25-hydroxycholecalciferol on the DNA–messenger RNA complex of the mucosal cell or an indirect alteration of the permeability characteristics of the nuclear membrane. Although the product of this nuclear or membrane interaction has been proposed to be a translocase or transport enzyme (*12*), the possibility that the substance formed is identical to the vitamin D dependent Ca BP isolated from the chick mucosa by Wasserman and co-workers (*5, 7, 9, 11*) must be entertained. Should this interpretation prove correct, alterations in vitamin D_3 metabolism in uremia (*3*) resulting in decreased intestinal 25-hydroxycholecalciferol concentrations could lead sequentially to decreased synthesis and concentration of the intestinal Ca BP and contribute to the defective calcium absorption characteristic of the chronic uremic state.

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References and Notes

1. M. M. Kaye, *J. Lab. Clin. Med.* **66**, 535 (1965); S. M. Genuth, U. Vertes, J. R. Leonards, *Metabolism* **13**, 124 (1969).
2. D. M. Kessner and F. H. Epstein, *Amer. J. Physiol.* **209**, 141 (1965).
3. L. V. Avioli, S. Birge, S. W. Lee, E. Slatopolsky, *J. Clin. Invest.* **47**, 2239 (1968).
4. J. W. Blunt, H. F. De Luca, H. K. Schnoes, *Biochemistry* **7**, 3317 (1968).
5. R. H. Wasserman, *Calcified Tissue Res.* **2**, 301 (1968).
6. D. Schachter, E. B. Dowdle, H. Schenker, *Amer. J. Physiol.* **198**, 263 (1960).
7. A. N. Taylor and R. H. Wasserman, *Arch. Biochem. Biophys.* **119**, 536 (1967).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. R. H. Wasserman, R. A. Corradino, A. N. Taylor, *ibid.* **243**, 3978 (1968).
10. A. Carlsson, *Acta Pharmacol.* **9**, 32 (1953).
11. R. H. Wasserman and A. N. Taylor, *J. Biol. Chem.* **243**, 3987 (1968).
12. J. E. Zull, E. Czarnowska-Misztal, H. F. De Luca, *Science* **149**, 182 (1965).
13. A. W. Norman, *Amer. J. Physiol.* **211**, 829 (1966).
14. Supported by PHS grants AM 06404 and AM 11674. L.V.A. is a PHS career research development awardee (7-K3-GM-22-676-03).

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Homology of Lactate Dehydrogenase Genes: E Gene Function in the Teleost Nervous System

Abstract. *Data obtained from immunochemical studies and the use of allelic variants support the hypothesis that the lactate dehydrogenase isozymes unique to the teleost nervous system are encoded in a third locus. Immunochemical and other studies demonstrate that this third locus is closely related to the lactate dehydrogenase B locus.*

Lactate dehydrogenase (LDH) exists in several isozymic forms in many organisms (*1*). The LDH polypeptide subunits in vertebrates are encoded in at least two codominant loci (*2, 3, 4*). The two corresponding types of polypeptide subunits produced are designated A and B (*2*). These subunits usually self-assemble randomly in vivo (*2, 4, 5*) or in vitro (*6*) to form the five tetrameric isozymes A_4, A_3B_1, A_2B_2, A_1B_3, and B_4 which are readily separable by electrophoresis.

Mammals and birds possess three LDH loci. The third locus, the C locus, functions only in primary spermatocytes (*7, 8, 9*). A third locus, referred to as the E locus (*10*), has also been postulated for teleosts by Markert and Faulhaber (*11*). The E locus, like the C locus, also exhibits great specificity of cell function (*10–16*). The E_4 isozyme, encoded in the E locus, usually possesses a very high net negative charge and is localized in the nervous system of teleosts, especially in the retina (*10–17*). However, in some species of teleosts the E_4 isozyme is also found in nonneural tissues such as lens (*12, 17*). On the basis of a comparison of the kinetic and physical properties of the teleost isozymes, I propose that the E_4 isozyme may be more closely related to the B_4 than to the A_4 isozyme (*17*).

The E subunits differ from the A and B subunits by hybridization in vivo (*10–14*) and in vitro (*10, 12, 14*). Especially revealing are the two types of E subunits produced in hybrid trout which are progeny of different parental species (*16*). Nevertheless these observations on subunit polymerization do not permit a critical conclusion concerning the genetic or epigenetic basis for the difference in subunit properties.

In order to clarify the genetic origin of the E subunit, allelic isozyme variants at the LDH B locus were examined in a panmictic population of killifish (*Fundulus heteroclitus*) from Woods Hole, Massachusetts. Figure 1 shows the LDH isozyme patterns of killifish eyes. The isozyme unique to the eye (E_4) migrates most rapidly toward the anode. The reversal of the relative electrophoretic mobility commonly observed in the A_4 and B_4 isozymes does not significantly alter their kinetic, physical, and immunochemical properties, which are homologous with those of the A_4 and B_4 isozymes of other vertebrates.

The three LDH phenotypes (Fig. 1) and their underlying genotypes responsible for alteration of B subunit mobility are slow (BB) and fast (B′B′), the two homozygotes, and the heterozygous hybrid (BB′) which possesses isozymes of intermediate mobility. The diallelic basis proposed for these phenotypes is supported by a population analysis of 245 adult killifish. The gene frequencies of the two *B* alleles were estimated