from the control patient C3. These differences in target cell survival were not the result of a nonspecific growth promoting effect by the control serum (C3) because a similar number of target cells remained after exposure to either T6 or C3 serum in the absence of lymphocytes. The induction of lymphocyte cytotoxicity by the T6 serum was dependent on concentration because further dilution of T6 serum (1/20) resulted in a diminution of the induced lymphocyte cytotoxicity against the TCC-bladder target cell (Table 1, experiment 1). Lymphocytes from most, but not all, lymphocyte donors were rendered cytotoxic to TCC-derived target cells by the LDA-like serum activity (Table 1, experiments 2 and 3). While lymphocyte cytotoxicity was induced against the TCC-bladder target cell line, normal kidney target cells tested simultaneously were not affected (Table 1, experiment 3). Thus the serum-induced lymphocyte cytotoxicity appeared to be antigen-specific (Table 2). Serum with LDA-like activity from patient T5 did not cause a significant reduction of either TCC-derived target cells or the normal kidney cells, in the absence of lymphocytes (Table 1, experiments 2 and 3).

While direct intrinsic lymphocyte mediated cytotoxicity against TCC target cells is also demonstrated [for example, lymphocytes from T1 versus lymphocytes from C2, in the presence of normal serum from C1, tested against TCC-bladder target cells (Table 1, experiment 3)], our method of analysis takes into account (by appropriate controls) the intrinsic cytotoxicity caused by the lymphocytes themselves; and thus the CI's (Table 1) are an estimation of the cytotoxicity induced by the presence of the LDA-like activity in serums from patients T5 and T6.

Studies to define target cell specificity were carried out with serum from patient T5. The results of 18 independent experiments demonstrate that serum from patient T5 (diluted 1 : 5) induced cytotoxicity in lymphocytes obtained from 87 percent of the TCC and control donors against the three TCC-derived target cell lines tested (Table 2). In addition, no cytotoxicity induction was detected against any of the normal target cells lines or against renal cell carcinoma tested simultaneously. Nine serum samples obtained at different dates from patients T5 and T6 also showed similar LDA-like activity in assays not presented here.

The cytotoxicity inducing activity of the serum factor (or factors) described above resembles the activity of lymphocyte dependent antibodies reported previously. Neither requires added complement, nor is either effective in the absence of effector (lymphoid) cells. Like previously described LDA (*3*), the serum factor demonstrated above appears to determine the specificity of the induced cytotoxicity. The LDA-like activity of serums from patients T5 and T6 appears to be directed against TCC (and not against HL-A antigens) since the cytotoxicity was induced against all the TCC cell lines tested but not against any of the seven non-TCC control cell lines (Table 2). While such target cell specificity suggests the participation of antibody, additional study will be required to determine whether this LDA-like factor is an IgG immunoglobulin.

It is not known whether thymus independent lymphocytes are the effector cells in these studies as they are in other LDA assays. This is significant because it has been reported recently that thymus independent lymphocytes are important in cell mediated cytotoxicity against human TCC-bladder cells (*12*). Thus, if the LDA-like activity of the TCC serums is similar to previously described LDA, it may potentiate the cytotoxicity of an effector cell type important in cell mediated immunity to human TCC.

The demonstration of the existence of serum dependent lymphocyte cytotoxicity against TCC's may provide an additional avenue for investigating the development and expression of immune responses against human solid tumors. If serum factors are capable of inducing in vivo lymphocyte mediated tumor destruction in man as they have been reported to do in animals (*13*), then perhaps the opportunity for immunotherapy in patients with transitional cell carcinomas may be enhanced.

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Partition of Tissue Functions in Epithelia: Localization of Enzymes in "Mitochondria-Rich" Cells of Toad Urinary Bladder

Abstract. *The mucosal epithelium of the toad urinary bladder reabsorbs sodium, acidifies the urine, and is responsive to neurohypophyseal hormones. Mucosal epithelial cells, consisting of two major morphologic cell types, "mitochondria-rich" and "granular," were removed from the bladder and separated by density gradient centrifugation. The mitochondria-rich cells contained three times as much carbonic anhydrase activity as the granular cells. Oxytocin caused a 235 percent increase in the adenosine 3',5'-monophosphate content of mitochondria-rich cells but had no effect on the granular cells. The evidence indicates that the mitochondria-rich cell, which accounts for only 15 percent of the mucosal cells, plays a major role in the mediation of sodium ion and hydrogen ion transport in the toad bladder and is a specific site of action of neurohypophyseal hormones.*

Epithelial membranes often contain several types of cells, each of which may contribute to different aspects of the tissues' overall physiology. Oxytocin, vasopressin, and related neurohypophyseal hormones cause an increase in the hydroosmotic permeability (*1*) of the urinary bladder of the toad, *Bufo marinus*, and an increase in the rate at which the tissue transports sodium (*2*) from the luminal surface to the nutrient surface. These responses are evidently mediated by a hormone-sensitive adenylate cyclase (*3*, *4*).

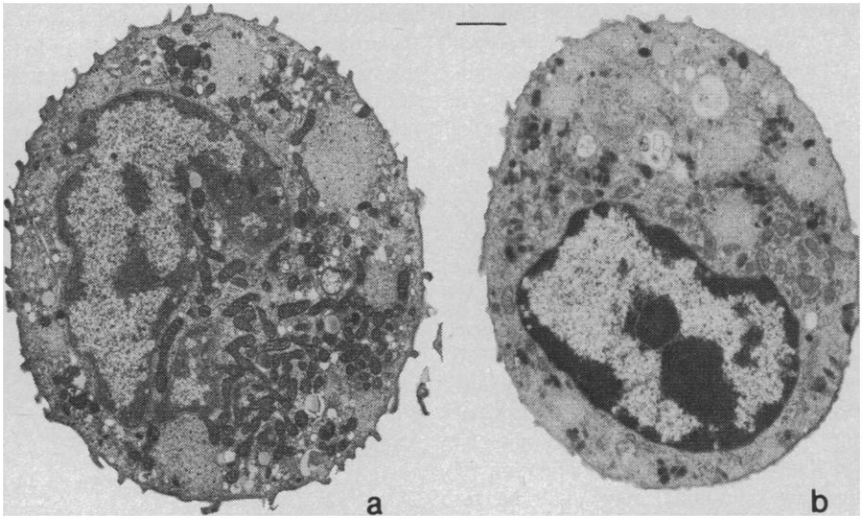

Fig. 1. (a) Typical mucosal cell (× 7000) recovered from band 2 (1.035 g/cm³) after centrifugation at 27,000 rev/min for 45 minutes. The cell contains many large mitochondria, and no granules are apparent. (b) Typical granular cell (× 7000) in material recovered from band 3 (1.067 g/cm³). Many electron-dense granules are present in the periphery of the cell. The mitochondria are not as numerous as in the cells from band 2 and their shape is more compact. Scale bar, 1 μm.

Under certain conditions, the bladder also acidifies the luminal fluid, a process which has been related to carbonic anhydrase activity (*5*). Electron micrographs of the mucosal epithelium show that it consists of two major morphologic cell types, "mitochondria-rich" cells and "granular" cells, which account for approximately 15 and 85 percent, respectively, of the cell population (*6*). Because it is not possible to directly measure the transport properties of any one cell or one population of cells, the different contribution of each type of mucosal cell to the transport of water and ions across the toad urinary bladder is not known. Nor is it known whether hormone responsiveness is limited to one population of cells. In two studies of this problem, coincident changes in cell morphology and in transport of sodium and water were examined to estimate the roles of the two major cell types (*7, 8*). As a more direct approach, we have attempted to separate the major morphologic cell types of the toad bladder mucosal epithelium. The responsiveness of the individual cell types to oxytocin and their carbonic anhydrase content were used as determinants of the contribution of each cell type toward the transport properties of the intact bladder.

Bufo marinus of Colombian origin (Tarpon Zoo Co., Tarpon Springs, Florida) and of Dominican origin (Lemberger Co., Oshkosh, Wisconsin) were used. Toads were pithed and the bladders were dissected and rinsed in Ringer solution. The necks of 12 to 16 hemibladders were individually tied to the outlet of a Luer-lock syringe, filled with and immersed in Ca^{2+}-free Ringer solution containing 2 m*M* ethylenediaminetetraacetic acid (EDTA), and incubated for 40 to 60 minutes at room temperature. The intraluminal fluid containing the disaggregated mucosal cells was removed and the cells were collected by centrifugation. The sedimented cells were resuspended in 10 ml of EDTA-Ringer solution and layered over a discontinuous gradient of Ficoll in EDTA-Ringer solution prepared in six centrifuge tubes (1 by 3½ inches). The gradient consisted of four solutions of Ficoll having densities of approximately 1.017, 1.035, 1.067, and 1.088 g/cm³ at 4°C. The cells were centrifuged at 27,000 rev/min in a Beckman SW-27 rotor for 45 minutes. After the centrifugation four bands of material were apparent. Each band of material was collected from the six tubes, pooled, suspended in 40 ml of EDTA-Ringer solution, and centrifuged. Tissue for transmission electron microscopy was prepared by adding the sedimented material to an equal volume of 6 percent glutaraldehyde buffered with 0.2*M* cacodylic acid (*p*H 7.3) for 2 to 6 hours. The fixed cells were rinsed in 10 percent sucrose, suspended in a 2 percent aqueous osmium tetroxide solution for 2 hours, and embedded in Epon 812.

The most buoyant layer, band 1, contained the smallest amount of material. This was composed almost entirely of heterogeneous acellular material and gave a positive periodic acid–Schiff reaction. This may represent the coating of mucus normally found on the luminal surface of the intact bladder. Bands 2 and 3 contained much more material, composed almost entirely of intact epithelial cells. The great majority of cells in band 2 were classified as mitochondria-rich (Fig. 1a). A rare goblet cell was identified, as well as an occasional granular cell. The cells in band 3 were primarily the granular type (Fig. 1b). These cells, which contained large numbers of the characteristic electron-dense

Table 1. Enzyme activities in toad urinary bladder mucosal cells separated by density gradient centrifugation. The data in each row are from one experiment, each using material from six to eight toads; values are per milligram protein. Cytochrome oxidase was assayed spectrophotometrically at 550 nm as the rate of oxidation of reduced cytochrome c. The ratio of cytochrome oxidase activity in the two bands differs from unity at $P < .001$. Carbonic anhydrase activity was measured by the method of Maren *et al.* (*13*); the difference in mean values for bands 2 and 3 is significant at $P < .005$. The means given are for both Colombian and Dominican toads.

Cytochrome oxidase activity (μmole/min)			Carbonic anhydrase activity (enzyme units)		
Band 2	Band 3	Ratio	Band 2	Band 3	Ratio
		Colombian toad			
142.5	119.7	1.19	3.21	1.35	2.38
128.2	112.9	1.14	1.98	0.77	2.57
114.9	95.7	1.20	1.68	0.52	3.23
157.1	132.7	1.18	2.37	1.22	1.94
			5.86	0.91	6.44
		Dominican toad			
77.0	69.8	1.10	3.05	0.97	3.14
51.7	42.7	1.21	3.01	0.96	3.14
45.3	39.0	1.16	1.21	0.50	2.42
Means		1.17 ± 0.02	2.80 ± 0.50	0.90 ± 0.11	3.16 ± 0.50

granules, also contained significant numbers of mitochondria. This band also contained an occasional small dense cell identified as a "basal" cell. Band 4, the most dense band (1.088 g/cm^3), contained cellular debris, as well as some intact epithelial cells, primarily granular cells.

To verify the morphologic evidence that we had separated the mitochondria-rich and granular cell types, we measured the cytochrome oxidase activity in the bands of separated material. Horse heart cytochrome c (Sigma Chemical Co., St. Louis, Missouri) was reduced with hydrogen over a palladium catalyst immediately before use. Isolated cells were suspended in 1.0 ml of Ringer solution and sonicated with a Branson sonifier for two 15-second intervals. Samples of this preparation were used to measure the rate of oxidation of cytochrome c in a Gilford absorption spectrometer at 550 nm (*9*). The cytochrome oxidase activity was consistently higher in band 2, the mitochondria-rich fraction of cells, than in band 3, which contained primarily the granular cells (Table 1). It was higher in both bands of mucosal cells prepared from toads of Colombian origin compared to toads from the Dominican Republic. The relative amounts of cytochrome oxidase activity in bands 2 and 3 were, however, identical in the toads from the two sources. These data lend support to our morphologic evidence that we had, to a large extent, separated the two major types of toad bladder mucosal cells.

It has been shown that under certain conditions the urinary bladder of the Colombian toad is capable of transporting H^+ into the urine (*5*). Because acidification is blocked by sulfonamide inhibitors of carbonic anhydrase, the process is presumed to be directly related to the activity of this enzyme. This enzyme is present in significant amounts in the mucosal epithelial cells and consists of at least three molecular forms (*10*). Histochemical studies of the toad urinary bladder, although perhaps lacking in specificity (*11*), have indicated that carbonic anhydrase is located primarily in the mitochondria-rich cells (*12*). Material collected by density gradient centrifugation was sonicated and centrifuged at 17,000 rev/min, and carbonic anhydrase activity was assayed in the supernatant solution by the method of Maren *et al.* (*13*). Carbonic anhydrase activity was approximately threefold higher in material from band 2 (mitochondria-rich cells) than in material from band 3 (granular cells) (Table 1). The distribution of the enzyme was apparently not related to the origin of the animal. Our data suggest that the sulfonamide-inhibitable transport processes leading to acidification of the urine are located primarily in the mitochondria-rich population of cells; this further corroborates our separation of the two cell types.

Table 2. Cyclic AMP content in basal and hormone-stimulated ($5 \times 10^{-8}M$ oxytocin) separated toad urinary bladder mucosal cells. Cyclic AMP was assayed by the radioimmunoassay method of Steiner *et al.* (*14*); values are per milligram protein. *N*, number of assays performed, each on tissue prepared from six to eight Colombian toads.

Cells	Cyclic AMP (pmole)	
	Band 2 ($N = 10$)	Band 3 ($N = 16$)
Basal	11.13 ± 0.56	12.69 ± 0.54
Oxytocin-treated	37.87 ± 2.11*	13.58 ± 0.45†

* Significant at $P < .001$. † Significant at $P > .2$.

To determine the oxytocin responsiveness of the two bands of cells collected by centrifugation, the cells were sedimented and resuspended in sodium Ringer solution containing 1.5 m*M* calcium. Portions of cells were added to 0.05*M* acetate buffer (*p*H 6.0) at 100°C before and 5 minutes after the addition of oxytocin ($5 \times 10^{-8}M$). After 5 minutes the cells were disrupted by sonication and centrifuged for 20 minutes at 5000 rev/min. The adenosine 3′,5′-monophosphate (cyclic AMP) content was measured in the supernatant fluid by the radioimmunoassay method of Steiner *et al.* (*14*), with commercially prepared materials (Collaborative Research, Waltham, Massachusetts). The labeled ligand was [^{125}I]succinyl cyclic AMP tyrosine methyl ester (600 c/mmole). The cyclic AMP–antibody complex was precipitated with goat antiserum to rabbit immunoglobulin G with a normal rabbit serum as carrier, collected by filtration (Millipore filter, 0.45 μm), and counted in a Nuclear-Chicago scintillation counter. The basal content of cyclic AMP in the two cell fractions was not significantly different (Table 2). However, following the addition of oxytocin to the cell suspensions, only the cells from band 2 (mitochondria-rich) showed a significant rise in cyclic AMP concentration. This increment in cyclic AMP amounted to 235 percent, significantly greater than the 145 percent increase in uncentrifuged mucosal cells under the same conditions (*4*).

Several observations have lent support to the possibility that the flux of water and sodium is not homogeneous through the mucosa of the toad urinary bladder. Civan *et al.* (*15*) found evidence for two parallel pathways for sodium movement through the tissue. Saladino *et al.* (*7*) found that the marked changes in active sodium transport following exposure of the toad bladder to amphotericin B were temporally related to morphologic alterations in the mitochondria-rich population of cells. DiBona *et al.* (*8*) presented evidence that vasopressin-induced osmotic water flow was limited to the granular cell. Our preparations of mitochondria-rich and granular cells represent significant enrichment of the two respective cell types in the banded material. Studies of biochemical events in these isolated cell types allow a more accurate determination of their individual roles in tissue function, such as in transport processes. Our data demonstrate that oxytocin-sensitive adenylate cyclase is located in the mitochondria-rich population. Moreover, carbonic anhydrase, another enzyme with a putative role in transport, is threefold more abundant in these cells. Our findings suggest that the mitochondria-rich cell is the locus of both H^+ transport and the initial step in oxytocin-stimulated transepithelial flux. The granular cell, which makes up most of the cell mass in the tissue, must certainly serve an important function, but in light of the evidence presented here the partition of cellular function in this tissue should now be reassessed.

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Genetic Regulation of Chlorophyll Synthesis Analyzed with Mutants in Barley

Abstract. *Barley seedlings homozygous both for the* xantha-l^{35} *and* tigrina-d^{12} *mutation accumulate magnesium protopophyrins and other precursors of chlorophyllide constitutively in darkness. The homozygous double mutant* xantha-f^{10}, tigrina-o^{34} *produces protoporphyrin constitutively. These results provide evidence for the control of chlorophyllide synthesis in higher plants through the products of regulatory genes in the nucleus.*

Among mutants in barley (*Hordeum vulgare* L.) that affect chloroplast development and greening, several *xantha* (*xan*) mutants and one *albina* (*alb*) mutant have blocks in chlorophyll synthesis (*1*, *2*). A number of *tigrina* (*tig*) mutants accumulate protochlorophyllide in darkness to a greater extent than does the wild type (*3*). The two groups of genes have been interpreted to be structural and regulatory genes, respectively, for chlorophyll synthesis (*3*). On this basis, certain predictions can be made about the phenotypes of double mutants containing both a structural and regulatory gene mutation. We now describe phenotypic characteristics of two double mutant genotypes.

Dark-grown barley seedlings accumulate in their etioplasts during the first 7 days a certain amount of protochlorophyllide, the immediate precursor of chlorophyllide. During further etiolation the quantity of protochlorophyllide declines (*4*). Illumination that results in the reduction of protochlorophyllide to chlorophyllide induces the further synthesis of protochlorophyllide. The following evidence supports the notion that the synthesis of protochlorophyllide from δ-aminolevulinate in etioplasts is regulated by repression and induction of an enzyme that catalyzes δ-aminolevulinate formation. Feeding the porphyrin precursor δ-aminolevulinate to dark-grown seedling leaves results in the accumulation of large amounts of protochlorophyllide and minor amounts of other porphyrins within the etioplasts (*2*, *5*), thus revealing that endogenous δ-aminolevulinate is the limiting factor in protochlorophyllide biosynthesis. The enzyme forming δ-aminolevulinate has a faster turnover than enzymes converting δ-aminolevulinate into protochlorophyllide (*6*). New synthesis of protochlorophyllide after an initial lighting is the result of the induction of δ-aminolevulinate synthesis (*7*, *8*). The induced synthesis of δ-aminolevulinate can be abolished by cycloheximide (*8*), an inhibitor of protein synthesis on cytoplasmic ribosomes (*9*).

While dark-grown structural gene mutants with blocked protochlorophyllide synthesis do not accumulate porphyrin precursors in the absence of exogenous δ-aminolevulinate, they pile up large amounts of different porphyrins and small or only trace amounts of protochlorophyllide when their leaves are supplied with δ-aminolevulinate (Table 1). Alleles at two structural gene loci were used in this investigation. Mutants homozygous for *xan-f*^{10} accumulate protoporphyrin, whereas mutants homozygous for *xan-l*^{35} produce in addition to protoporphyrin large quantities of magnesium protoporphyrin and its monomethyl ester (*2*) (Fig. 1, c and d). We infer provisionally that the *xan-f* gene codes for a protein participating in the insertion of magnesium into protoporphyrin IX and that the *xan-l* gene codes for a protein catalyzing a step between magnesium protoporphyrin monomethyl ester and protochlorophyllide. Since mutant seedlings do not accumulate these porphyrins unless supplied with exogenous δ-aminolevulinate, we conclude that the repression of δ-aminolevulinate synthesis is not affected by the lesions. This is supported by measurements of δ-aminolevulinate pools in the mutants (*8*). Induction of δ-aminolevulinate synthesis upon illumination does not occur in the almost completely blocked mutant *xan-f*^{10}, but takes place to some extent in the leaky *xan-l*^{35} (*8*). The latter result indicates that the photoreduction of protochlorophyllide to chlorophyllide a is a prerequisite for the induction of δ-aminolevulinate synthesis by light.

The *tigrina* mutants listed in Table 2 under regulatory genes accumulate amounts of protochlorophyllide in the dark, which exceed those produced in the wild type by 1.5 to 15 times. The four mutant alleles in the *tig-b* gene differ in the amount of protochlorophyllide produced in the seedling leaves.

Table 1. Structural genes involved in chlorophyll biosynthesis in barley. Data on the origin and genetics of these lethal and sublethal mutants are found in (*10*).

Structural genes	Mutant alleles		Phenotype of homozygous mutant seedlings in the dark	
	Number	Nature	Porphyrins accumulated upon feeding δ-aminolevulinate (*2*)	Carotene content (*11*)
xan-f	8 (2 leaky)	Recessive	Protoporphyrin	
xan-g	5 (2 leaky)	Recessive	Protoporphyrin	
xan-h	4	Recessive	Protoporphyrin	
xan-l	1 (leaky)	Recessive	Mg-protoporphyrins + protoporphyrin	
xan-u	1 (leaky)	Recessive	Protoporphyrin + uroporphyrinogen	Blocked in β-carotene synthesis, accumulate aliphatic polyenes
alb-e	1 (leaky)	Recessive	Protoporphyrin	Reduced content