mutability term dependent upon amino acid composition and the mutabilities of individual amino acids. (Mutability here means the frequency with which evolutionary substitutions occur and is broader than the term in its strict genetic sense.) Such a correlation was suggested by the second explanation for rates of protein evolution. In general, a large number of considerations need to be taken into account for a detailed analysis of the possible replacements for an amino acid: Interior hydrophobic groups may be replaced by other hydrophobic groups; exterior polar groups may be replaced by other polar groups; the size of an interior side chain would restrict the number of amino acid side chains that could replace it; amino acids in a structural element (helix, β sheet, turn) may be replaced by amino acids either favoring the same structure or at least compatible with it.

To perform such an analysis for an individual protein and determine the number of acceptable replacements for each amino acid is certainly a difficult task. There is, however, a set of empirical values that would seem to approximate the results of a detailed analysis. These values are the relative mutabilities of the amino acids, which are proportional to the ratio of the number of accepted mutations of an amino acid over the number expected if all amino acids were equally likely to have acceptable replacements (*2*). Values calculated for individual families of proteins are similar to the average relative mutabilities calculated for all the protein families considered (*2*). It appears, then, that the empirical value, the average relative mutability of an amino acid, will approximate the result of a detailed analysis of possible replacements.

From the relative mutability (*2*) (m_i) of each amino acid and its fractional occurrence (f_i) in a protein, it is possible to calculate a mutability term ($\Sigma m_i f_i$) for that protein. I shall call this term the total relative mutability of a protein and have calculated its value for a number of proteins. The larger this term, the greater the number of amino acid replacements (per unit length of protein) that could occur without disrupting the overall structure of the protein. Consequently, the greater this mutability term, the faster the rate at which mutations are accepted in the evolution of a protein sequence (given that overall structure is maintained and that mutations in general occur randomly and uniformly with time). This expectation is borne out by Fig. 1, which plots the rates of sequence evolution against the total relative mutability. The overall correlation is good; statistical analysis gives $P < .01$ and a Pearson correlation coefficient (r) of .58. Of the 24 proteins tested, 20 follow the correlation quite well ($P < .001$, $r = .85$); they lie in a band about the regression line, $y = 1.92x - 130$.

The four proteins that deviate most markedly are growth hormone and amyloid protein A (above the main trend) and glucagon and glyceraldehyde-3-phosphate dehydrogenase (below the main trend). There may be special reasons why these are exceptional. At least two of them are exceptional in other ways; for example, the rate of evolution of growth hormone is not constant when different sets of mammals are compared (*8*), and the sequences of human amyloid protein A differed by 8 of 76 amino acid positions when determined for two different subjects (*9*).

Whatever the explanation for these exceptional proteins, they are only a fraction of all the proteins examined. For most of the proteins there is a strong relation between the rate of evolution and a mutability term dependent upon amino acid composition. This relationship was predicted by, and is compatible with, one possible explanation for the variability of rates of protein evolution, namely, that fairly stringent structural constraints apply to most proteins and rates of evolution reflect the number of structurally acceptable replacements for the amino acids of a protein.

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17 December 1974; revised 25 February 1975

Immunofluorescent Localization of Cyclic AMP in Toad Urinary Bladder: Possible Intercellular Transfer

Abstract. *By use of an immunofluorescent cytochemical staining technique, adenosine 3′,5′-monophosphate (cyclic AMP) has been localized in toad bladder epithelial cells. Within 2 minutes after addition of vasopressin, staining intensity increases in both mitochondria-rich and granular cells. This finding, taken together with the precise anatomieal relation between these two epithelial cell types and the observation that after separation of the two cell types vasopressin stimulates cyclic AMP accumulation in only mitochondria-rich cells, suggests that cyclic AMP may be transferred from mitochrondria-rich to granular cells as part of the response of the toad urinary bladder to vasopressin.*

The hormone-sensitive epithelial cell layer of the toad urinary bladder consists of two principal cell types: a mitochondria-rich (MR) cell, whose surface is covered by microvilli, and a cell containing numerous electron-opaque cortical granules, whose surface is covered by arborizing ridges (granular cell). These two cell types are arranged in a precise anatomical relationship; each MR cell is surrounded by four or five granular cells, and these anatomical units cover the entire epithelial surface (*1–3*). Addition of arginine vasopressin (AVP), a posterior pituitary antidiuretic hormone, produces several striking changes in the structure and function of the epithelial cell layer; there is an immediate increase in both water permeability and transepithelial sodium transport (*4*), both MR and granular cells swell (*1*), and the surface processes of the granular cells elongate and develop microvilli (*3*). These changes are accompanied by increased tissue adenosine 3′,5′-monophosphate (cyclic AMP) content (*5*). However, when epithelial cells are separated by Ficoll density gradient centrifugation into MR and granular cell fractions, an increase in cyclic AMP can be demonstrated only in the MR cell fraction after addition of AVP to the isolated cells (*6*). This apparent discrepancy in cellular localization between the physiological and biochemical response of the toad bladder epithelium to AVP suggested that cyclic AMP might be transferred from MR to granular cells and prompted our immunofluorescence localization study of cyclic AMP in intact toad bladder.

The immunofluorescent technique has

been described (*7*). We used cryostat sections (~ 10 μm thick) of unfixed tissue previously incubated in the absence (control) or presence of AVP. After air-drying on glass slides, sections were incubated at 22°C in a moist chamber under a 1 : 8 dilution of the immunoglobulin G (IgG) fraction of rabbit antiserum to cyclic AMP. After a 10-minute reaction, sections were quickly washed in phosphate-buffered saline, *p*H 7.5, and fixed for 5 minutes in freshly dissolved 2 percent paraformaldehyde. The bound rabbit IgG against cyclic AMP was then localized by incubation of sections with fluorescein isothiocyanate-conjugated goat IgG prepared against rabbit IgG (Miles Laboratories). After this second reaction, slides were rinsed, mounted with cover slips, and then examined by dark-field fluorescence microscopy utilizing epiillumination through an FITC (fluorescein isothiocyanate) filter with a 150-watt xenon lamp and a 500-barrier filter. In previous studies it has been demonstrated that this technique is specific for cyclic AMP (*7, 8*). Staining is blocked by preincubation of rabbit IgG against cyclic AMP with cyclic AMP, but is not interfered with by preincubation with other adenine or cyclic nucleotides.

As shown in Fig. 1A, positive immunofluorescence is seen over both epithelial cells and muscle. Within 2 minutes after addition of AVP (Pitressin, 65 munit/ml; Parke, Davis) there is increased staining intensity for cyclic AMP localized over both epithelial cells and smooth muscle (Fig. 1B). The increased immunofluorescence over epithelial cells appears uniformly distributed over all cells, not localized to either MR or granular cells. Increased staining is not, however, seen over vascular or stromal elements. After a 12-minute exposure to AVP the cytoplasm of all epithelial cells stains intensely, leaving only the centrally located nuclei nonreactive (Fig. 1C). After 20 minutes in the presence of AVP the degree of staining has diminished and the tissue again resembles control sections (Fig. 1D). Thus, there appears to be no selectivity with regard to cell type for localization of cyclic AMP in toad bladder epithelial cells after AVP addition. Additionally, the time course of the change in immunofluorescence we observed after AVP addition parallels the changes in cyclic AMP chemically measured in both whole tissue (*5*) and unseparated epithelial cells after hormone addition (*9*).

As support for the validity of the immunofluorescence technique for localizing cyclic AMP in toad bladder, we have stained

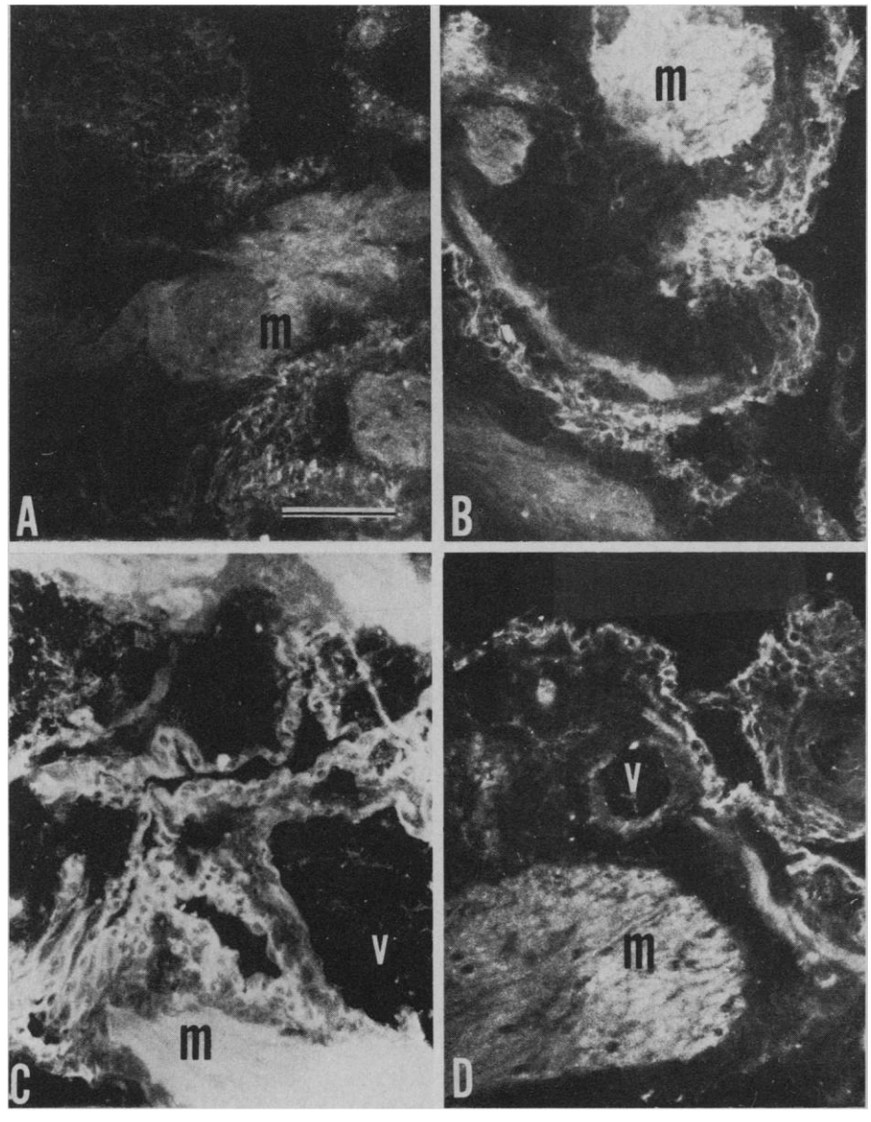

Fig. 1. Dark-field fluorescence micrographs of toad urinary bladder stained for cyclic AMP. Cut strips of hemibladder were incubated in Ling-Ringer phosphate, *p*H 7.4 (*11*). At the appropriate time after addition of AVP, strips were removed and frozen as spirals for cryostat sectioning and preparation as described in the text. All fields were exposed for identical periods, and the resulting negatives printed, exposed, and developed for the same durations. (A) Control. Positive staining is seen over both smooth muscle (*m*) and epithelial cells (calibration bar, 50 μm). (B) Two minutes after AVP addition. Increased staining intensity is seen over both epithelial cells and smooth muscle. In the epithelial cell layer all cells in a given area stain with the same intensity. (C) Twelve minutes after AVP addition. The cytoplasm of all cells in the epithelial cell layer stains very intensely. The endothelium of a blood vessel (*V*) embedded in connective tissue also stains lightly. (D) Twenty minutes after AVP addition. Epithelial cell staining is reduced but still appears more intense than control (A).

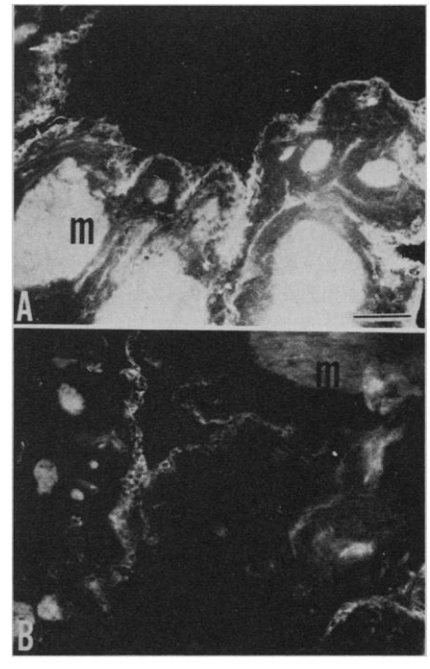

Fig. 2. Dark-field fluorescence micrographs of toad bladder stained for cyclic AMP after incubation in (A) a sodium-free sucrose buffer and (B) a sodium-containing Ling-Ringer phosphate buffer (*11*). Deletion of sodium results in intense staining of both epithelial cells and muscle (*m*).

sections of tissue incubated for 30 minutes in a buffer where sucrose replaces isosmotically the sodium present in the usual Ling-Ringer phosphate buffer (*10*). Under these experimental conditions, tissue cyclic AMP concentrations are increased fivefold over control values (*11*). As shown in Fig. 2, there is a striking increase in the immunofluorescence seen in the tissue on deletion of sodium from the buffer. The increase in staining, however, is most prominent over muscle, although the epithelial cells also stain more intensely.

The results reported here demonstrate that the cyclic AMP content of all epithelial cells, both granular and MR, increases in intact toad bladder after AVP addition, and that both the biochemical and the physiological effects of AVP are manifest not only in MR but also in the more prevalent granular cells. This result contrasts sharply with the results obtained with separated MR and granular cells (*6*). If the latter results are valid—that is, if the hormone responsiveness of granular cells has not been destroyed during separation of granular and MR cells—then the present results raise the possibility that there may be a rapid and specific transfer of cyclic AMP from MR to granular cells. The transfer would not appear to be extracellular as there is no evidence for leakage of immunofluorescent staining material out of the epithelial cells. The repeated rosette arrangement of four or five granular cells surrounding a central MR cell (*3*) could thus provide the proper anatomical relation for intercellular chemical transfer. While as yet there is no direct evidence for such intercellular communication, the work reported here, as well as previous work by Loewenstein *et al.* (*12*) demonstrating low-resistance electrical pathways between toad bladder epithelial cells, raises this possibility.

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21 October 1974; revised 10 December 1974

Neural Plasticity in Visual Cortex of Adult Cats after Exposure to Visual Patterns

Abstract. *Over a period of 2 weeks, adult cats were twice a day exposed for 1 hour to a visual environment consisting only of vertical stripes and for the rest of the time were kept in darkness. Subsequent investigation of the striate cortex showed a decrease in the number of neurons sensitive to orientations around the vertical relative to those sensitive to horizontal orientations. This indicates that plasticity of functional properties of the cortical neuronal network still exists in adult animals.*

Most of the neurons of the visual cortex of all mammals so far investigated respond best or exclusively if moving contrasts, especially light or dark lines, are used as stimuli. An individual neuron responds optimally only to a specific orientation of the line and a specific direction of movement, the latter being usually normal or nearly normal to the optimal orientation. Most neurons have an orientation "tuning width" of 10° to 30° around the optimal orientation and do not respond at all to stimuli oriented 30° to 90° away from their optimal stimulus orientations (*1*). In adult animals, at least, all orientations are represented equally frequently within the whole neuronal population (*1, 2*). If young kittens are raised, during the critical period from 3 to 14 weeks of age, in a visual environment consisting only of stripes of one orientation, most neurons will be maximally sensitive to orientations within about ±30° of the one to which they were exposed (*3*). A similar effect has not yet been reported in adult animals.

We have done experiments on seven adult cats (more than 1 year old, weight 2.2 to 3.4 kg). The animals were kept for 14 days in a completely dark cage. Twice a day they were put into a cylinder (2 m high, 60 cm in diameter) for 1 hour. The inner wall of the cylinder was covered with vertical stripes of variable widths between 0.5° and 7.5°, measured from the center of the cylinder. The cat was sitting in the middle of the cylinder on a horizontal partition made of clear plexiglass so that the vertical stripes were continuous in the upper and lower visual field. The cylinder was brightly lit from above through a diffusing plexiglass lid, resulting in a brightness of 17 cd/m² of the white stripes and 1.4 cd/m² of the dark stripes. The animals were observed from time to time through a small hole in the lid.

Four to 12 hours after the last exposure to this visual environment, the animal was anesthetized in the dark with Brevimythal (25 mg/kg) intraperitoneally, and its eyes were carefully covered. The further preparation followed the standard procedure of this laboratory (tracheotomy and artificial respiration, trepanation of the skull over the visual cortex and fixation of a closed chamber, widening of the pupils with atropine, and retraction of the nictitating membrane with Neo-Synephrine). The refraction, which was determined with an ophthalmoscope, was corrected with contact lenses. Except during receptive field testing, the eyes were covered with a black mask. Stimuli were projected onto a screen 1 m in front of the eyes. After muscle relaxation with 2 ml of Flaxedil, an infusion containing 1.0 ml of Flaxedil, 0.3 ml of glucose solution, and 1.7 ml of Ringer solution was continued throughout the experiment (3 ml/hour). The electroencephalogram, electrocardiogram, CO_2 level, and temperature were continuously monitored. All animals were kept under N_2O respiration (70 percent N_2O, 30 percent O_2) throughout the experiment. Recording from single units was done with glass microelectrodes (filled with 1*M* NaCl). Area 17 was penetrated near the midline so that recordings from many "columns" were possible during one penetration. When a unit was found, one eye was uncovered and the receptive field and trigger feature were