ceptive fields in the binocular segment of visual field, and the monocular segment of these structures is similarly defined. For example, in the geniculate, the binocular segment includes the medial, laminated portion including all of lamina A1 and corresponding parts of the A and C laminae; the monocular segment is the lateral region of the nucleus where the A and C laminae extend beyond lamina A1.
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6 June 1974

Aldosterone-Induced Protein in Toad Urinary Bladder

Abstract. *Simultaneous electrophysiological and biochemical experiments demonstrated a specific aldosterone-induced protein in paired urinary hemibladders isolated from the toad* Bufo marinus. *Whenever aldosterone stimulated short-circuit current, aldosterone specifically increased [^{35}S]methionine incorporation into a low-molecular-weight protein (about 12,000). Comparative studies with dexamethasone and insulin and inhibitory studies with spironolactone and actinomycin D suggest mineralocorticoid specificity.*

Aldosterone and other mineralocorticoids stimulate transepithelial sodium transport across the isolated toad urinary bladder (*1*). This stimulation is characterized by a lag of 30 to 90 minutes before sodium transport increases, and by sensitivity to inhibition by actinomycin D, puromycin, and cycloheximide (*2*). The stimulation is not produced by glucocorticoids (*1*) and can be abolished by spironolactones which displace aldosterone from its nuclear binding sites (*3*). On the basis of these data and by analogy with other model systems of steroid hormone action, one widely held hypothesis (*4*) suggests that aldosterone acts within the cell nucleus to induce the synthesis of a specific RNA, which then codes for the synthesis of a specific protein (aldosterone-induced protein; AIP). This protein, which may be a membrane component, a permease, or a carrier molecule, is responsible for the increased sodium transport induced by the hormone. However, there has not yet been any direct proof of this hypothesis; neither a specific aldosterone-induced RNA (*5*), nor a specific AIP (*2*, *4*) has been demonstrated. This report presents evidence from toad urinary bladders (i) that aldosterone specifically induces the synthesis of a low-molecular-weight protein or polypeptide and (ii) that the production of this AIP is correlated with the stimulation of sodium transport.

Female toads (*Bufo marinus*) were obtained from the Dominican Republic and maintained on 0.6 percent NaCl solution for 48 hours before use. The paired, excised hemibladders were placed in continuously aerated, doubly buffered Ringer solution (DBR; see below) at room temperature (22° to 24°C) for 15 to 30 minutes before measurements were begun. The standard doubly buffered Ringer solution (DBR) consisted of 98.2 mM NaCl, 3.4 mM KCl, 4.9 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 2.5 mM $NaHCO_3$, and 5 mM dextrose, along with penicillin (50 unit/ml), streptomycin (50 μg/ml); and phenol red (0.01 percent) as *p*H indicator. The *p*H was 7.9 to 8.1, and the osmolality was 210 to 220 milliosmoles per kilogram.

A modified Lucite double chamber was used for the electrophysiological studies (*6*). One of each pair of hemibladders was mounted across the entire chamber, so that a single hemibladder provided an experimental and a control quarter bladder. Each quarter-bladder isolated its own serosal and mucosal compartments (cross-sectional areas of 2.3 cm^2). The transepithelial potential difference and the short-circuit across each quarter-bladder were used to measure sodium transport (*1*, *6*). After a stable baseline was obtained (for both potential difference and short-circuit current), *d*-aldosterone (Calbiochem) was added (in a methanol carrier) to the serosal medium for the experimental quarter-bladder (usual final concentration, $10^{-6}M$); the serosal medium (5 ml) for the control quarter-bladder received the same volume (10 μl) of the methanol carrier (without aldosterone). (Similar results can be obtained with aldosterone concentrations of $\backsim 10^{-7}M$; a concentration $> 10^{-8}M$ is usually needed to stimulate short-circuit current.)

Each hemibladder studied biochemically was monitored with an electrophysiological experiment performed on the corresponding hemibladder from the same toad, incubated under the same experimental and control conditions.

After one of each pair of hemibladders was removed for the electrophysiological studies described above, the corresponding hemibladder was cut into two to four parts for biochemical studies. Each piece of tissue (wet weight, $\backsim$ 80 mg) was incubated at 24° to 25°C with 2 ml of DBR containing [^{35}S]methionine (Amersham/Searle) and the specific additions were indicated for each experiment. In most studies the standard DBR was modified by adding nine or four parts of DBR to one part of $NaHCO_3$-free, methionine-free, minimal Eagle's medium (MEM; Microbiological Associates), and was adjusted to *p*H 7.9 to 8.0 with tris buffer.

After incubation, the epithelial cell layer was removed by scraping, washed twice with cold DBR, and collected by centrifugation at 600*g* for 1 minute. This washed pellet was then resuspended in 1 ml of lysing buffer (*p*H 8.5) consisting of 0.1M glycine, 0.1M NaCl, 0.01M EDTA, 0.1 percent sodium dodecyl sulfate (SDS), and 0.01M β-mercaptoethanol. The suspension was then frozen and thawed successively, three times, and dissolved by heating at 50°C with agitation for 1 hour, and then at 100°C in a water bath for 10 to 15 minutes. The solution was centrifuged, and the residue was extracted twice with lysing buffer at 100°C. Little residue remained after this procedure. Four volumes of cold acetone were added to the supernatant, and the solution was allowed to stand

overnight at −20°C. The precipitate was collected by centrifugation and resuspended in 0.5 ml of buffer (*p*H 6.8) containing 0.05*M* tris, 1.0 percent SDS, 10 percent glycerol, and 1 percent β-mercaptoethanol. The suspension was dissolved at 100°C (5 minutes). Approximately 1 to 2 mg of protein was recovered (*7*) from the epithelial layer from one quarter-bladder. (A sample of the protein was precipitated with 10 percent trichloroacetic acid, and the radioactivity of the acid-insoluble material was assayed on nitrocellulose filters.)

The radioactive proteins of the lysate were analyzed by discontinuous gradient, cylindrical, and slab gel electrophoresis (*8*), and by autoradiography (*9*). The relative optical density of the developed film was determined with a Joyce-Loebl double-beam scanning microdensitometer.

The time course of [^{35}S]methionine incorporation into trichloroacetic acid–precipitable material obtained from epithelial cells scraped from the toad bladder was linear over a 4-hour period, from 105 (at 1 hour) to 432 (at 4 hours) count/min per microgram of protein. The autoradiograms from the aldosterone-treated and control epithelial cell lysates (Fig. 1A) show that the rates of polypeptide synthesis (measured by grain density of the x-ray film after 3 hours of incubation with [^{35}S]methionine) are almost identical, except for an increase (see →) in synthesis of a low-molecular-weight polypeptide or protein, or both. In the densitometric tracing of these gels (Fig. 1B), the corresponding peaks in the low-molecular-weight region are 2.4 (optical density units) for the aldosterone-treated cells, and 1.9 for the controls. The corresponding paired hemibladder demonstrated a clear aldosterone response (more than a 100 percent increase in short-circuit current, compared to control).

Similar results were obtained with a nonautoradiographic method of analysis. Corresponding short-circuit currents and amino acid incorporations obtained from another experiment are shown in Fig. 2, where gels from control and aldosterone-treated preparations were sliced, and the radioactivity in each slice was assayed. When compared to the control, aldosterone induces a discrete increase in [^{35}S]methionine incorporation into new protein or polypeptide (or both) at slice 42 (Fig. 2A). No other slice showed a consistent difference between experimental and control preparations. The short-circuit current data from the paired hemibladders (Fig. 2B) show a typical aldosterone response.

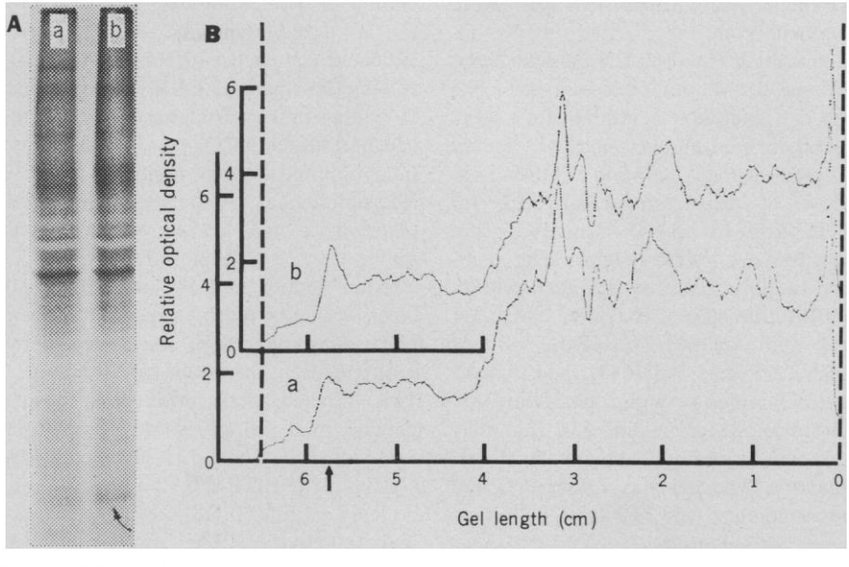

Fig. 1. Effect of aldosterone on the incorporation of methionine into protein in an aldosterone-responsive bladder. Two hemibladders were excised from a single toad, and prepared as described. One hemibladder was studied electrophysiologically (not shown), while the other hemibladder was divided in two parts: one of which was incubated in 2 ml of DBR with 10^{-6}*M* *d*-aldosterone (Calbiochem), while the other was incubated without aldosterone. After equilibrium at 24°C for 20 minutes, [^{35}S]methionine (∽ 10 μc/ml; 60 c/mmole) was added. The incubation was continued for an additional 4.5 hours before the scraped cells were prepared for electrophoresis. Approximately 70,000 count/min (for both experimental and control preparations) were placed on a slab gel for electrophoresis and autoradiographic analyses (control, 9.8×10^5 count/min per milligram of protein; aldosterone, 12.4×10^5 count/min per milligram). (A) Autoradiogram: a, control; b, aldosterone. (B) Densitometric tracings of autoradiogram. The abscissa is common for both tracings, but the ordinates are offset.

The correspondence of an aldosterone-induced increase in short-circuit current to an aldosterone-induced increase in radioactivity (count/min) in the region of the low-molecular-weight protein was observed in ten separate paired hemibladder experiments. It is unlikely that these specific results derive from aldosterone-induced changes in the size of the methionine pool, unless the induced protein is the only one with a short half-life. In four additional paired experiments, the short-circuit currents for the aldosterone-treated quarter-bladders were equal to those of the controls, and the corresponding experimental and control quarter-bladders exhibited no differences in amino acid incorporation.

There are at least three reasons why this aldosterone-induced difference in the low-molecular-weight region has not been observed previously. First, the low-molecular-weight region of traditional 7.5 or 10 percent SDS gels (*10*) does not afford the resolution of the present techniques; the traditional gels are most suited for resolution of higher-molecular-weight proteins. Second, if the specific activity of our total protein mixture (200 to 1000 count/min per microgram of protein) were one-tenth that actually used, this low-molecular-weight material (2 to 3 percent of the total) would represent at most 100 to 200 count/min. Consequently, the low-molecular-weight region would not be resolved adequately by either autoradiography or by traditional acrylamide gel slicing techniques. (This high specific activity could not be achieved in our preliminary experiments with [^{14}C]- and [^{3}H]leucine.) Third, the toad bladder exhibits marked individual variability in aldosterone-responsiveness. If electrophysiological controls from the paired hemibladders were not used, the pooled biochemical responses would not have revealed the differences.

The effects of four other agents were evaluated to establish the specificity of the increased [^{35}S]methionine incorporation into a low-molecular-weight

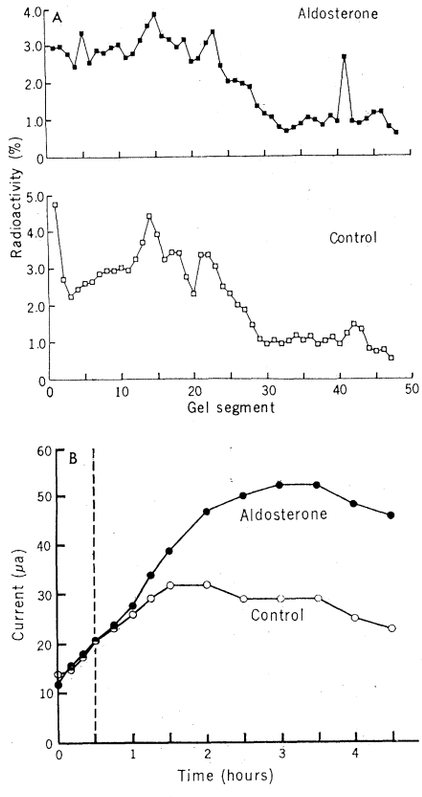

Fig. 2. Effect of aldosterone on methionine incorporation into protein in an aldosterone-responsive bladder. The incubation medium contained [^{35}S]methionine (15 μc/ml; 180 c/mmole) in 2 ml of DBR. Approximately 80,000 count/min of each sample was placed on the slab gel for electrophoresis: control, 2.51 $\times$ 10^6 count/min per milligram of protein; aldosterone ($10^{-6}M$), 2.72 $\times$ 10^6 count/min per milligram of protein. (A) Gel slice radioactivity. Samples from aldosterone-treated and control tissues were placed on a gradient (7.5 to 25 percent) cylindrical acrylamide-gel (length, 10 cm; inner diameter, 6 mm) and subjected to electrophoresis at 75 volts for 16 hours. The gels were sliced and dissolved in 30 percent hydrogen peroxide. The radioactivity is expressed as a percentage, that is, (the counts per minute per slice) $\times$ 100 divided by the sum of the number of counts per minute in the gel. In contrast to the large difference at slice 42, the small change at slice 5 in the aldosterone-treated tissue was not reproducible in other experiments. (B) Aldosterone-induced short-circuit current. The absolute short-circuit current is plotted as a function of time. The vertical dashed line indicates the time at which aldosterone ($10^{-6}M$) was added to the experimental quarter-bladder, while the methanol carrier was added to the control. This hemibladder (two paired, quarter-bladders) was obtained from the same animal as the hemibladder shown in (A).

protein for aldosterone: dexamethasone, insulin, spironolactone, and actinomycin D.

1) Since high concentrations of several steroid hormones increase the incorporation of RNA precursors into RNA and of amino acids into protein (*11*), the specificity of the aldosterone-induced change in the low-molecular-weight region was tested by comparing the effects of a mineralocorticoid (aldosterone, $10^{-6}M$) with those of a glucocorticoid (dexamethasone, $10^{-6}M$) and a control in the same tissue. Although aldosterone exerted its stimulatory effect on the low-molecular-weight region, and the corresponding hemibladder exhibited typical short-circuit current response to aldosterone, the same concentration of dexamethasone failed to induce similar autoradiographic changes in tissue from the same animal (four experiments). These data suggest that "mineralocorticoid" rather than "glucocorticoid" effects are correlated with the new protein synthesis.

2) Since insulin stimulates sodium transport across the toad urinary bladder with a time course similar to that of aldosterone stimulation (*12*), we performed analogous experiments to those described above, but used insulin (1 milliunit/ml) instead of dexamethasone. In four experiments, insulin increased both the short-circuit current and the specific incorporation of [^{35}S]methionine into AIP. In each of these experiments aldosterone also increased the specific incorporation of [^{35}S]methionine into AIP. For similar degrees of stimulation of short-circuit current, there were similar increases in the incorporation of [^{35}S]methionine into AIP. In contrast to aldosterone, however, insulin also increased the overall incorporation of [^{35}S]methionine into total protein.

3) Spironolactones inhibit the actions of aldosterone (*3*, *4*). Spironolactone ($10^{-4}M$) was added 20 minutes before aldosterone ($10^{-7}M$) and compared to aldosterone alone ($10^{-7}M$) and a control. Spironolactone addition blunted both the electrophysiological (increased short-circuit current) and biochemical (increased [^{35}S]methionine incorporation into AIP) responses to aldosterone.

4) The effects of actinomycin D were studied because of its known inhibitory actions on aldosterone-induced sodium transport and on RNA metabolism (*2*, *4*, *5*). Paired hemibladders were incubated in actinomycin D (2 μg/ml) for 3 hours in 50 ml of DBR. After being washed one hemibladder was divided into three parts. Each part was incubated separately for 3 hours in modified DBR: (i) control, (ii) with aldosterone ($10^{-6}M$), and (iii) with both aldosterone ($10^{-6}M$) and actinomycin D (2 μg/ml). Actinomycin D suppressed the biochemical response to aldosterone in this tissue, although corresponding tissues responded to aldosterone (after the actinomycin was washed out) both biochemically and electrophysiologically (*13*, *14*).

In order to characterize the molecular weight of the aldosterone-induced protein or polypeptide (AIP), the epithelial cell lysates were directly compared to a preparation of known proteins on the same gels and autoradiograms. On this basis the AIP appears to have a molecular weight of about 12,000, and may be a protein, polypeptide, or protein subunit.

Since only prolonged exposure to actinomycin D prevented the increased synthesis of AIP in competent tissue, aldosterone may not act solely at the transcription step of protein synthesis. The precise relation between the increase in the synthesis of AIP and the increase in net sodium transport in aldosterone-sensitive tissue is unclear, and more specific data would be required to demonstrate any stoichiometric or causal relation between the concentration of AIP and net sodium transport.

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13. When similar studies were performed with 30 minutes of exposure to actinomycin D, with or without aldosterone addition, there was increased synthesis of AIP (*14*). Although prolonged exposure to actinomycin D can inhibit hormone-induced new protein synthesis in many tissues by preventing transcription, brief exposures to actinomycin D (with or without hormone) may actually result in increased synthesis (superinduction) of a particular protein (*15*). Since others also have evidence that such may be the case in toad urinary bladders (*16*), actinomycin D may have a posttranscriptional effect on protein synthesis.
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9 May 1974; revised 27 June 1974 ■

Hearing with the Third Ear: Dichotic Perception of a Melody without Monaural Familiarity Cues

Abstract. *Julesz has shown that cross-correlations between two patterns that appear random to either eye alone can give rise to the perception of form and depth when viewed stereoscopically. We produced auditory analogs by presenting eight simultaneous and continuous sine waves to both ears and by either phase-shifting or frequency-shifting one of them relative to its counterpart in the opposite ear. Particular tones were shifted in sequence such that a melody was heard which was undetectable by either ear alone.*

Julesz (*1*) has shown that if one presents a field of random dots to one eye and the same field to the other eye, but with a small portion shifted horizontally, a certain area of the percept appears to stand out in depth. Its contour is the boundary of the shifted portion of dots, and the shift is logically impossible to detect by one eye alone. Julesz named this phenomenon *cyclopean* perception, after the mythical beast that looked out at the world through a single eye in mid-forehead. With random-dot stereograms it is possible to bypass, as it were, the peripheral visual apparatus and project information to the cyclopean eye and onto "the 'mind's retina'—that is, at a place where the left and right visual pathways combine in the visual cortex" (*1*, p. 3). Our goal was to devise an auditory analog to the cyclopean percept, one which for etymological reasons we call *cyclotean* (*2*), in which the peripheral auditory apparatus is bypassed and information is projected onto the "mind's cochlea."

We were provoked into seeking this goal, in part, by Julesz (*1*, p. 51) and Julesz and Hirsh (*3*), who claim that analogies between visual and auditory perception are not "very deep." The basis for their view is that visual perception is primarily concerned with spatial *objects* whereas auditory perception is primarily concerned with temporal *events*. The distinction between objects and events appears to be primarily founded on the potential richness of percept in each modality: two spatial dimensions are possible for a visual percept, whereas only the one temporal dimension is available for an auditory percept.

In pre-cyclopean days, Huggins and later Cramer and Huggins (*4*) demonstrated that if one presents white noise to one ear and the same white noise to the other, but with a narrow band of frequencies time-delayed, a faint pitch quality is heard. It sounds like narrow-band noise, and is logically impossible to detect with one ear alone. Nevertheless, it fails to meet Julesz' criterion for an auditory object: It is "not a truly cyclopean phenomenon since the input variable is a single time delay, while the perceived variable is a single pitch" (*1*, p. 51). Julesz elaborates by stating that to create a true analog to the visual phenomenon with such pitches one would need to generate a melody. Since a melody is a pattern of pitches and varies in both time and frequency, it is multidimensional and hence an auditory object. Thus, we decided to produce a cyclotean melody. In order to probe the generality of auditory analogs we chose to generate it by two conceptually distinct methods. The first is a methodological offshoot of Cramer and Huggins and is analogous to existing visual work; the second method, on the other hand, is wholly new.

In both demonstrations the basic stimulus consists of eight, simultaneous, continuous, computer-generated sine waves whose frequencies were chosen from the even-tempered scale in the key of G (*5*). In both, the tune "Daisy" was embedded in the tonal arrays as a cyclotean melody. All stimuli were computed numerically, generated by the PCM system (*6*) at Haskins Laboratories, and recorded at the same time on two channels of audio tape.

Our first demonstration begins with the presentation of the basic stimulus to both ears, by earphones, with a lag of 1 msec between the onsets of the two inputs (input A leading input B). A constant discord of eight tones is heard for 1500 msec. At that point the first note, D_5, is introduced by advancing the phase of the D_5 component of input B by 1 msec and by delaying the phase of its counterpart in input A by the same amount. The phase-shifting process is not instantaneous, but occurs over a 45-msec duration. The first note is sustained by maintaining the new phase relation until 900 msec have elapsed after the initiation of the phase shift, at which time the phase-shifting process is reversed (again taking 45 msec) until the two corresponding sine waves resume their original phase relationship. The offset phase-shifting of D_5 completely overlaps with the onset of the subsequent note, B_4. Subsequent notes in the tune are introduced and removed in the same fashion. The duration of each note is from 112.5 msec for an eighth note to 1800 msec for a double-whole note. The duration of the entire sequence is approximately 24 seconds. A spectral segment of it is represented in the top panel of Fig. 1.

Subjectively, the melody is perceived to occur inside the head but displaced to one side of the midline, while a background noise is localized to the