

stock grazing and the creation of a large buffer zone around ERDA's Hanford facilities has encouraged a large native ungulate to establish itself in a historically unoccupied range.

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4. Aerial surveillance takes place at weekly intervals to maintain fence integrity and to discourage off-road vehicle trespass.
5. Research conducted by Battelle, Pacific Northwest Laboratories for the Energy Research and Development Administration under contract E(45-1)-1830.

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Cytochrome c: Immunofluorescent Localization of the Testis-Specific Form

Abstract. *Mouse testes contain a unique form of cytochrome c. As demonstrated by the indirect immunofluorescence technique, the testis-specific cytochrome c is detectable in the primary spermatocyte and in cell types comprising the later stages of spermatogenesis. Interstitial cells, Sertoli cells, and spermatogonia contain the somatic form of cytochrome c, as does heart muscle.*

The germinal epithelium of mature testes is a highly specialized tissue designed for the production of spermatozoa. This tissue has many unique features, not the least of which is the presence of a number of proteins that are synthesized only in cells committed to spermatogenesis (1). The best example of such a protein is the testis-specific form of lactate dehydrogenase (LDH-C₄ or LDH-X; E.C. 1.1.1.27). This isozyme is composed of four C subunits, distinct from the A and B subunits found in other tissues (2). It first appears in the primary spermatocyte dur-

ing midpachytene (3) and ultimately becomes the predominant if not the sole LDH of sperm. It now appears that an analogous phenomenon can be observed with cytochrome c, the electron-transport protein of the mitochondrial respiratory chain.

Hennig (4) recently reported the isolation of two different cytochromes c from mouse testes. One is identical to the cytochrome c found in mouse heart, while the other protein differs from the first by approximately 13 residues in the amino acid sequence (4). We shall refer to these

molecules as cytochrome c_s, for the protein presumably found in all tissues, and cytochrome c_t for that form isolated from testes. In this report, we demonstrate that mouse cytochrome c_t is strictly confined to spermatogenic elements of the seminiferous epithelium.

Approximately 40 mg of pure cytochrome c_t was prepared from 1.9 kg of testes dissected from about 10,000 sexually mature, random-bred mice (5). Antiserums were raised in male New Zealand White rabbits by injecting them with glutaraldehyde-cross-linked polymers of either cytochrome c_s or cytochrome c_t (6). The specificity of the serums was tested by double diffusion in agar gels (7). The antiserum to cytochrome c_s reacted strongly with cytochrome c_s and weakly with cytochrome c_t. Conversely, the antiserum to cytochrome c_t reacted much more strongly with cytochrome c_t than cytochrome c_s. The cross-reacting antibodies in antiserum to cytochrome c_t were removed by absorption on a column consisting of cytochrome c_s bound to Sepharose 4B (8). The absorbed serum reacts only with cytochrome c_t and not cytochrome c_s. Because of the limited amount of cytochrome c_t available, it was not possible to obtain an antiserum to cytochrome c_s that did not cross react to a slight extent with cytochrome c_t.

Localizations of the cytochromes c were performed by the indirect fluorescent antibody technique (9) on sections cut from paraffin-embedded tissue which had been fixed in Bouin's fluid. After reaction with rabbit antiserum to cytochrome c, the slides were washed, treated with fluorescein isothiocyanate conjugated goat antiserum to rabbit immunoglobulin G, and examined by transmitted light dark-ground-fluorescence microscopy (10). Appropriate controls showed that the fluorescence in the sections treated with antiserum was due solely to specific interaction with the cytochromes c, and not to fluorescence of the tissue itself or to fluorescence induced by nonspecific binding of rabbit gamma globulins to the mouse tissue.

Immunofluorescent analysis of mouse testis from a sexually mature animal revealed that cytochrome c_t was confined to cells of the germinal epithelium. The antibody reaction appears as a discrete granular fluorescence in the cytoplasm, strongly suggestive of mitochondrial localization (Fig. 1A). From examination of adjacent sections stained with hematoxylin and eosin, the cells closest to the tubule periphery which are positive for cytochrome c_t are primary spermatocytes. Spermatogonia which occupy the basal

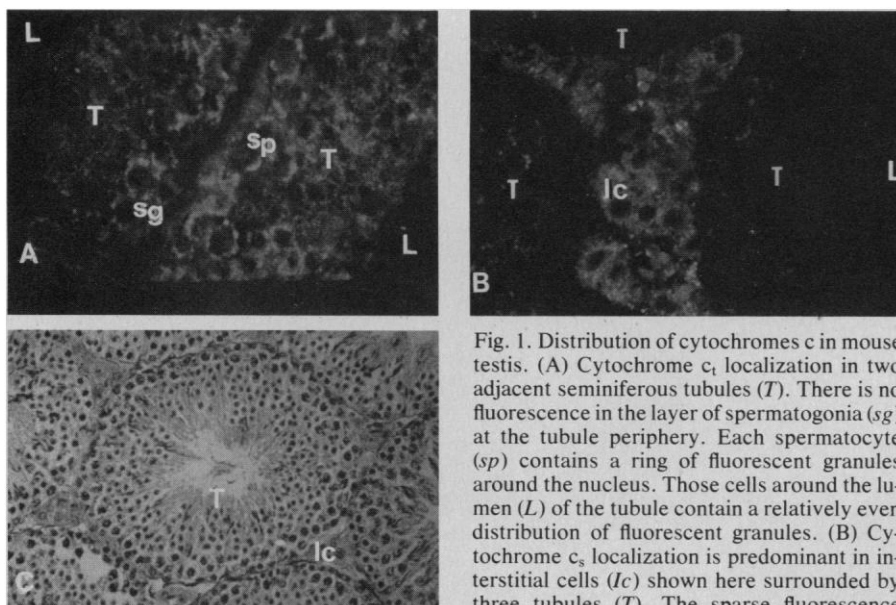


Fig. 1. Distribution of cytochromes c in mouse testis. (A) Cytochrome c_t localization in two adjacent seminiferous tubules (T). There is no fluorescence in the layer of spermatogonia (sg) at the tubule periphery. Each spermatocyte (sp) contains a ring of fluorescent granules around the nucleus. Those cells around the lumen (L) of the tubule contain a relatively even distribution of fluorescent granules. (B) Cytochrome c_s localization is predominant in interstitial cells (Ic) shown here surrounded by three tubules (T). The sparse fluorescence

within tubules may be associated with Sertoli cells and spermatogonia. There may also be some cross-reaction with cytochrome c_t (see text). (C) Low-power photomicrograph of a section of testis stained with hematoxylin and eosin, illustrating the orientation of tubules (T) and interstitial cells (Ic).

layer of the tubules, and Sertoli cells, do not react with antibody to cytochrome c_1 . Around the large nucleus of the primary spermatocytes a ring of mitochondria fluoresce in response to treatment with antiserum to cytochrome c_1 . In secondary spermatocytes and spermatids the same granular fluorescence tends to be more evenly distributed throughout the cytoplasm, as expected from the known distribution of mitochondria in these cells (11).

A strikingly different pattern of fluorescence is produced by antibodies to cytochrome c_s . As shown in Fig. 1B, the cytochrome c_s is localized in interstitial tissue, Sertoli cells, and in what appear to be spermatogonia along the tubule periphery. Only a faint fluorescence is seen in spermatocytes and spermatids.

To confirm that the immunofluorescent technique effectively localizes cytochrome c_s , sections of mouse cardiac muscle were examined. The mitochondria in this tissue fluoresced brightly after treatment with antiserum to cytochrome c_s (Fig. 2).

These observations provide direct evidence that the cytochrome c_1 of testis is confined to those cells comprising the germinal epithelium. The absence of the protein from spermatogonia and its clear localization in the primary spermatocyte suggest that the corresponding gene is abruptly activated as the cell commits itself to maturation. Nongerminal elements, such as interstitial cells and Sertoli cells, appear to have only cytochrome c_s . The low levels of fluorescence seen in the germinal elements with antiserum to cytochrome c_s may be due to the cross-reactivity of this serum with cytochrome c_1 , or to small amounts of cytochrome c_s in these cells. When sufficient mouse cytochrome c_1 is accumulated for immunoadsorption, it will be possible to obtain antiserum monospecific for cytochrome c_s , and to employ the two monospecific antisera to estimate the relative proportions of cytochrome c_1 and cytochrome c_s at each stage of spermatogenesis. This should make it possible to determine whether cytochrome c_1 is simply added to mitochondria already containing cytochrome c_s , whether some mechanism exists for exchanging the cytochromes within the mitochondrion, or whether a new population of mitochondria containing only cytochrome c_1 is generated.

The localization of testicular cytochrome c in cells comprising the germinal elements of the testes is remarkably similar to the distribution of LDH-C₄. This enzyme is restricted to mature, sperm-producing testes of mammals and

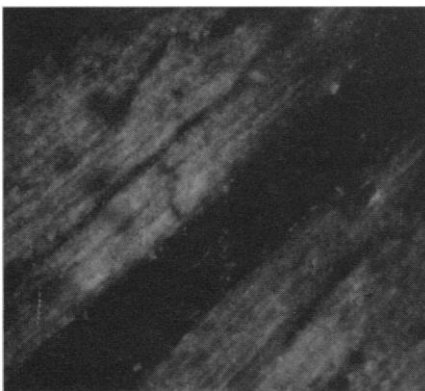


Fig. 2. Immunofluorescent localization of cytochrome c_s in the mouse heart. The localization of fluorescent granules approximates the distribution of mitochondria among the muscle fibers.

birds and is the product of a structural gene first active in primary spermatocytes (2). Conversely, the more ubiquitous isozymes of LDH composed of A and B subunits are synthesized in most somatic tissues (12).

The occurrence of multiple proteins carrying out the same reaction in a single organism exemplifies the specialization permitted by gene duplication. The LDH isozymes have been used as a model of the evolution of duplicate genes (13). Thus, the somatic tissue LDH isozymes are the products of two genes, *Ldh-a* and *Ldh-b*, which probably are monophyletic in origin. Each tissue synthesizes different proportions of A and B subunits to meet its own metabolic requirements. Limited available evidence favors the suggestion that the third gene, *Ldh-c*, which codes for LDH-C₄, arose by tandem duplication of the *Ldh-b* gene (14), and subsequently evolved to be specifically activated in spermatogenic cells. Similarly, a single form of cytochrome c , cytochrome c_s , predominates in all organisms examined, and its line of descent throughout the eukaryotes has been established by comparisons of amino acid sequences (15). Presumably, this gene was duplicated at some time, and one copy diverged to cytochrome c_1 . From this single example, it is not possible to determine in which ancestral group of species the duplication event occurred.

The divergence of the *Ldh-c* locus has been accompanied by evolution of a regulatory mechanism that permits the synthesis of the lactate dehydrogenase C subunit only during spermatogenesis (2). Whether expression of the gene for cytochrome c_1 is similarly controlled is yet undetermined. In a survey of pig tissues (16) only cytochrome c_s was found in heart, liver, brain, kidney, and skeletal

muscle. With specific antisera, a detailed examination can be undertaken of many tissues in both sexes as well as of very early developmental stages.

The role of such testis-specific gene products as LDH-C₄ and cytochrome c_1 in the germinal epithelial cells committed to sperm formation must be ascertained. They must fulfill important metabolic functions either during spermatogenesis or in the sperm itself. Additionally, the unique gene products in these highly specialized cells provide sensitive molecular probes of the processes underlying differentiation.

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9. Paraffin sections were rinsed for 2 minutes each in two changes of xylol, then 100, 95, and 80 percent alcohol, and distilled water. All sections were then rinsed in phosphate-buffered saline, pH 7.3, three times for a total of 10 minutes, and finally dipped in deionized water. Tissues were blotted and excess moisture allowed to evaporate. Antiserum to cytochrome c_1 or to cytochrome c_s was applied to the tissue, and the slides were incubated in a moist chamber for 30 minutes at room temperature. Slides were then rinsed for a total of 10 minutes in three changes of phosphate-buffered saline, dipped in deionized water, and blotted as above. Fluorescein isothiocyanate conjugated goat antiserum to rabbit gamma globulin (FITC-gARGG; Cappel Laboratories) at a 1:2 dilution was then layered on the sections. Incubation and washing was done as above. The slides were mounted in 90 percent glycerin and 10 percent phosphate-buffered saline (pH 8.0), covered with No. 1 cover slips, and examined immediately.
10. A Zeiss fluorescent microscope and camera attachment with a halogen lamp, dark-field condenser, excitation filter KP-500, and a series of barrier filters was used to examine and photo-

- graph all fluorescent slides. The barrier filters employed and the exposure times for photography were varied according to the intensity of fluorescence. The photographs of Figs. 1 and 2 were obtained under exactly comparable conditions of exposure.
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Cerebrospinal Fluid Production: Stimulation by Cholera Toxin

Abstract. Large increases in the production of cerebrospinal fluid have been observed after the intraventricular administration of cholera toxin. Because cholera toxin stimulates adenylate cyclase, the data suggest that adenosine 3',5'-monophosphate plays a role in cerebrospinal fluid production.

The ability of adenosine 3',5'-monophosphate (cyclic AMP) to mediate hormonal responses in various secretory epithelia, including the kidney, salivary glands, intestinal epithelia, pancreas, and thyroid, has been well documented (1). Purified cholera toxin serves as a specific adenylate cyclase-cyclic AMP probe. After it is introduced to the luminal side of epithelia, it stimulates adenylate cyclase production of cyclic AMP and subsequent electrolyte transport in the gut (2, 3) and the kidney (4). Recent studies have also shown that cholera toxin can stimulate endolymph production in the inner ear (5).

Cerebrospinal fluid (CSF) is secreted by the choroid plexus and associated ventricular structures and absorbed primarily through the arachnoid villi (6). This secretion has been directly correlated with transepithelial flux of electrolytes; however, the biochemical mechanism is still poorly understood. We have now demonstrated the ability of intraventricularly administered cholera toxin

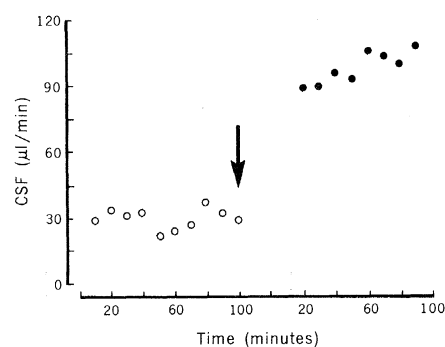


Fig. 1. Production of CSF in a representative experiment before (○) and after (●) the intraventricular administration of cholera toxin. The arrow indicates the time the toxin was introduced into the ventricle. There was a 2-hour incubation period before the perfusion was started again. Production of CSF was determined by inulin dilution.

to stimulate cerebrospinal fluid secretion, ostensibly through stimulation of an adenylate cyclase-cyclic AMP pathway in the choroid plexus and periventricular compartment.

Experiments were performed on mongrel dogs weighing 20 to 25 kg, anesthetized with pentobarbital, and maintained on positive pressure ventilation. Arterial pressure, heart rate, intracranial pressure, body temperature, p_{O_2} , and p_{CO_2} (the partial pressures of O_2 and CO_2 , respectively) were constantly monitored and stabilized. Cerebrospinal fluid secretion was measured through the use of a modification of the method of Pappenheimer *et al.* (7). Both lateral ventricles were perfused with Elliotts B artificial CSF containing 2 μ C of [^{14}C]carboxy-inulin (New England Nuclear) per 100 ml at a rate of 0.15 ml per minute per ventricle; a pump (Harvard model 2219) and two 50-ml syringes in parallel were used. The outflow catheter was placed at zero pressure with respect to the ear so that bulk collection of fluid represented CSF production plus the volume of the perfusate less the amount absorbed. Cerebrospinal fluid production was calculated by the indicator dilution method (8). After the values of normal CSF formation were determined for each dog, the perfusion was stopped and 250 μ l of purified cholera toxin (100 μ g per milliliter of normal saline), heat-inactivated cholera toxin, or saline was injected into each lateral ventricle. Cholera toxin was prepared as previously described (5). After 2 hours of incubation, the perfusion was again started and CSF formation was again measured. Statistical analyses were made between baseline and experimental values with *t* tests.

The effects of cholera toxin on CSF production by a typical dog are shown in Fig. 1. Figure 2 shows a significant dif-

ference ($P < .001$) between control production (47.0 ± 7.0 μ l/min) and secretion after cholera toxin challenge (102.0 ± 8.0 μ l/min); however, no significant difference was observed between control rates (59.0 ± 6.0 μ l/min) and rates after exposure to saline (59.0 ± 6.0 μ l/min) or between control rates (57.0 ± 4.0 μ l/min) and production after incubation with heat-inactivated cholera toxin (68.0 ± 7.0 μ l/min). Measurement of the volume of CSF released from the cisternal catheter showed similar increases after cholera toxin challenge, as was also seen in the isotope dilution technique; these results indicate that cholera toxin did not change brain permeability to inulin. Control secretion was 17.0 ± 6.0 μ l/min, and secretion after cholera toxin challenge was 73.0 ± 7.0 μ l/min. There was no significant change in the volume from the cisternal catheter after incubation with saline or with inactivated cholera toxin.

Normal values reported for CSF formation in this report are similar to those shown previously with the inulin-dilution technique. The higher production found with inulin dilution as compared with bulk flow at the cisternal catheter is a function of CSF absorption through normal pathways and inulin diffusion into the brain (9). Inactivated cholera toxin was used as a control to demonstrate that injected protein does not significantly contribute to increased fluid production (Fig. 2). The possibility that cholera toxin was absorbed and acted system-

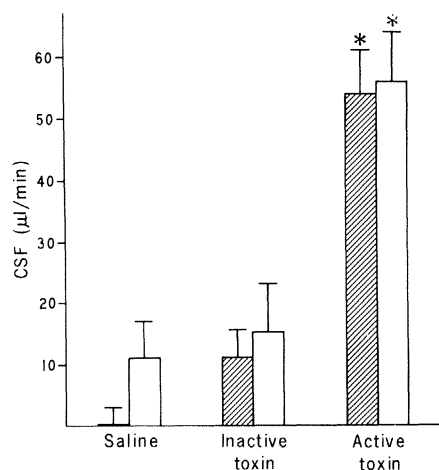


Fig. 2. Differences between baseline and experimental CSF production after intraventricular administration of saline, heat-inactivated cholera toxin, and active cholera toxin. Shaded bars represent change in CSF formation calculated according to the inulin dilution technique, and open bars represent the change in bulk collection at the cisterna magna outflow catheter. Brackets represent the standard errors of the mean; $N = 5$ in all groups. Asterisks indicate a significant difference ($P < .001$).