# Basic Aspects of Cystic Fibrosis

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## Introduction

Cystic fibrosis (CF) is manifestly a disease of the exocrine organs *(1)* to which the respiratory, digestive, and reproductive tracts are hereby included as honorary members. In years past, few individuals with CF survived infancy. Up to 10% of affected children died shortly after birth as a result of complications from meconium ileus, and survivors, who were typically malnourished, usually succumbed to repeated and persistent lung infections. These problems can now be ameliorated, but the underlying defect remains (Fig. 1).

In CF, the lungs lose their ability to maintain a sterile surface and are gradually destroyed by ineradicable colonies of bacteria, typically *Pseudomonas*, which convert to a mucoid form *(2)*. The intestinal lining appears to secrete less fluid than normal and is, therefore, susceptible to blockade from improperly dehydrated stools *(3–5)*. This can lead to meconium ileus or its equivalent, but the tendency for stools to be dehydrated is often more than offset by the presence of steatorrhea, which is secondary to reduced secretion of pancreatic fluid *(6–8)*. Additional symptoms that are almost invariably present include the blockage and eventual degeneration of the vas deferens in males *(9)*, dehydrated cervical mucus and a failure of the mucus to show appropriate hydration during ovulation in females *(10)*, and greatly elevated concentrations of $Na^+$ and $Cl^-$ in the sweat *(11,12)*.

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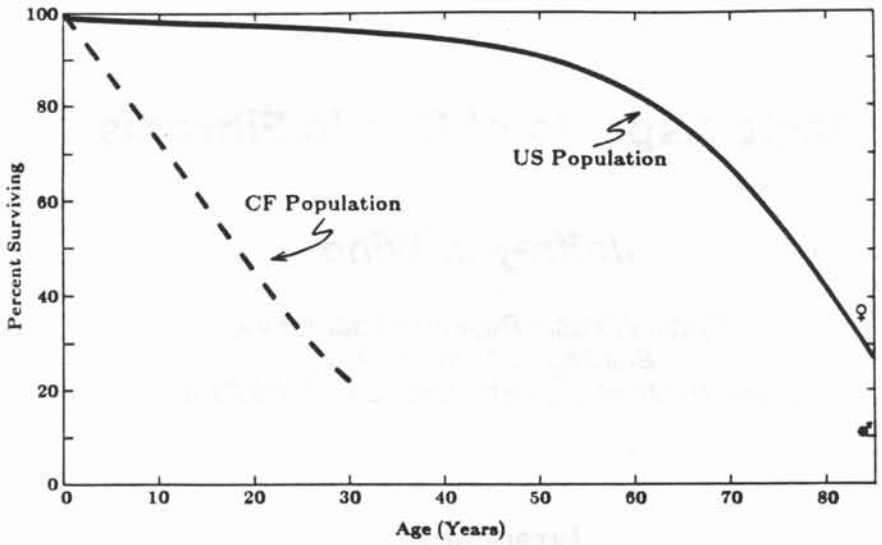


Fig. 1. Survival curves, US, CF vs entire population. The CF curve is based on ref. *78*. Age of 50% survival has since increased and is probably now in mid-twenties. Symbols on ordinate at right indicate survival at age 85 for black males and white females.

Symptoms variably present include focal biliary cirrhosis and an unusually small gallbladder that may also have small mucus-filled pockets in its epithelium *(13)*. Gallbladder and liver involvement each may be present in as many as 25% of CF individuals, but are not correlated with one another nor with general clinical status *(13)*. Comprehensive texts treat most aspects of the clinical syndrome (e.g., *14,15)*.

The defect in all organs that are invariably affected is correlated with, and may be caused by, a fundamental abnormality in the way the epithelial cells that line these organs secrete and absorb salty fluids *(16,17)*. The defects that have been shown most definitively are a marked reduction in $Cl^-$ permeability in several epithelia *(18–22)*, and a failure of at least one epithelium to secrete fluid in response to β-adrenergic stimulation *(23)*. The molecular explanation for these defects is now close at hand, for after a heroic search the gene responsible for CF was recently identified *(24–26)*.

## *The CF Gene*

The CF gene *(24–26)* comprises about 250,000 base pairs within region q31 of chromosome 7 (Fig. 2). The expressed portion of the gene, amounting to only 2–3% of the total gene, is distributed among at least 24 exons. These code for a protein of 1480 amino acids. This is the only gene that causes CF, but several different alleles exist. In the most common defective allele (70% of CF alleles in North America), 3 adjacent nucleotides have been deleted from near the middle of the coding region, causing a corresponding deletion of the 508th amino acid from the *N*-terminus of the protein. The deleted amino acid is phenylalanine (F), and the allele is designated $DF_{508}$. The number of alleles that account for the remaining 30% of CF genes is not yet known, but the number is probably large. New alleles are being identified rapidly.

Allelic differences are likely to account for some of the variable symptoms found in CF *(26)*. When all alleles have been identified and a comprehensive test has been devised, CF clinicians will be able to obtain the exact allelic profile of CF patients in their care. Careful clinicians, who have always tailored treatments on the basis of empirical data, should be able to use the allelic profiles of their patients to refine therapy further.

Given the evidence for multiple alleles, it remains puzzling that CF is such a common genetic defect among Caucasians, whereas its occurrence is rare in other groups. About 3% of Caucasians carry the $\Delta F_{508}$ allele, and about 1.5% carry the remaining alleles *(26)*. Perhaps it is only the $\Delta F_{508}$ deletion allele that is specific to Caucasians. If that allele were not present in a population, but all remaining CF alleles were present in equivalent proportions, the incidence of CF would be reduced to about 1/18,000 individuals. The analysis of Kerem et al. *(26)* indicates that 25% of the non-$\Delta F_{508}$ alleles lead to a mild form of CF and that the mild allele is dominant. In a population lacking the $\Delta F_{508}$ allele, only about 1/40,000 individuals would display the severe form of CF. This figure does not differ much from some estimates of CF in non-Caucasian populations *(27)*. If the above speculation is valid, it is the prevalence of the $\Delta F_{508}$ allele in Caucasian populations that will need to be explained. It might be a founder effect, perhaps coupled with and amplified by a heterozygote advantage *(28,29)*.

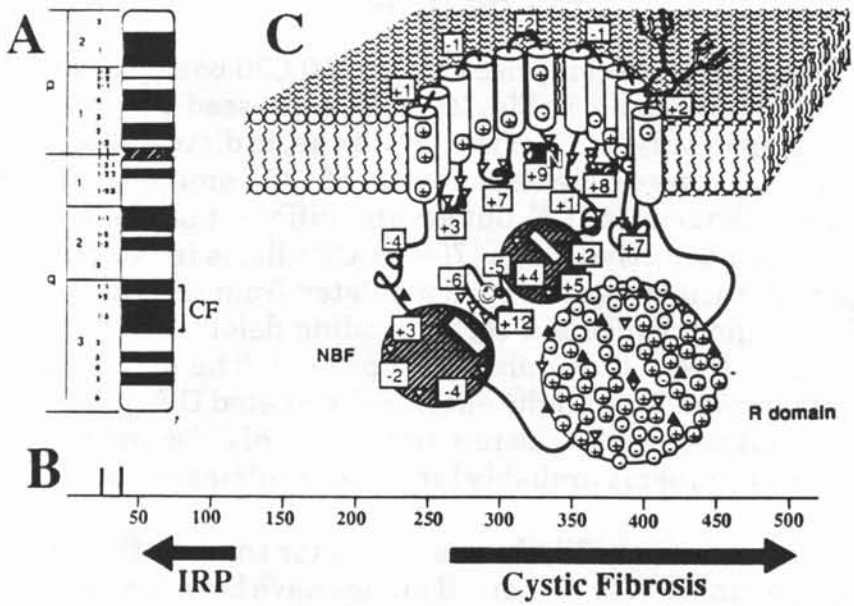


Fig. 2. The CF gene and its products. **A.** The gene was localized to region q31 of chromosome 7 by RFLP mapping techniques. **B.** Approximate position of the gene. Scale is in kilobases. IRP shows the location of a gene mapped to near CF in 1987 (*79*). **C.** A model of the product of the CF gene: The CF transmembrane conductance regulator protein (CFTR). The protein consists of 1480 amino acids, with a predicted arrangement of transmembrane helices as shown. Shapes of the cytosolic and extracellular domains are arbitrary. N and C termini are indicated by open letters; charged amino acids are indicated individually for the transmembrane helices and the R domain, and by totals (in boxes) for other domains. Downward pointing open triangles are consensus sequences for phosphorylation by cAMP-dependent protein kinase; upward pointing filled triangles are consensus sequences for phosphorylation by protein kinase C. The two darker, spherical domains contain nucleotide binding folds (NBFs); a phenylalanine in the NBF domain of the N terminal half of the protein is deleted in proteins made by the major CF allele *(from 25)*.

## *The CF Gene Product: The CF Transmembrane Conductance Regulator (CFTR) Protein*

The product of the CF gene has been named the CF transmembrane conductance regulator protein (CFTR) (*25*). From analysis of the cDNA sequence, the protein is predicted to be membrane-bound, with 12 membrane-spanning domains. According to the present model, only about 2% of the polypeptide backbone is extracellular,

17% is in the membrane, and about 80% is exposed to the cytosol. A striking feature of the model is that it consists of two domains of very similar overall structure, but with only modest sequence similarity. Each domain comprises 6 putative transmembrane helices and a large, cytosolic nucleotide binding domain or "fold" (NBF). The longest extracellular loops are between the first and 2nd helices of each domain, with the other loops being short (maximum of 6 amino acids). The cytosolic loops between the 2nd and 3rd transmembrane helices in each domain are of virtually identical length (56 and 58 amino acids), as are those between the 4th and 5th helices (66 and 68 amino acids). For each domain the NBFs are of similar length (154 and 168 amino acid residues) and are located about the same distance from the C-terminal transmembrane helix (82 and 68 amino acids in each "pre-NBF" loop). The two domains are linked by a large, highly charged cytosolic domain of 241 amino acids, termed the "R" domain, that includes 16 potential sites for phosphorylation by cAMP-dependent protein kinase and protein kinase C *(25)*.

The preponderance of cytosolic domains, the dual sites for binding ATP, the plethora of potential phosphorylation sites, and the large number of transmembrane helices, including amphipathic helices, are consistent with the molecule being regulated via cytosolic factors and participating in transmembrane transport. The evidence presented below supports a close association between the CFTR and the movement of $Cl^-$ across membranes, but it is not yet clear how such movement is effected nor whether $Cl^-$ transport is the only direct function of the CFTR. The intense study of the CFTR protein now underway should produce results of general biological interest, extending far beyond the domain of CF.

The tissue distribution of the CFTR has been assessed by searching for expression of its mRNA in various human tissues and cell lines *(25)*. Transcripts were found in lung, nasal polyp, pancreas, colon, a colonic tumor cell line (T84 cells), kidney, placenta, liver, parotid gland, and cultured sweat gland cells, but not in brain, adrenal gland, nor in cell lines of skin fibroblasts and lymphoblasts. This analysis is now being complemented with antibodies to the protein to provide a detailed understanding of the cellular and subcellular distribution of the CFTR protein.

It may turn out to be of great interest that the CFTR protein is similar to the P-glycoprotein, or multi-drug resistance protein, which

is often overexpressed when certain cancerous cells are treated with anti-cancer drugs intended to kill them or block their multiplication *(30)*. In cells that have been selected for drug resistance, it is found that cytosolic levels of the drugs are very low, and there is now good evidence that the P-glycoprotein pumps the drugs back out across the cell membrane before they can harm the cell. Some normal cells also express detectable amounts of P-glycoprotein. It is interesting that such cells include epithelial cells that line the pancreas, liver, kidney, and intestine, and that in such cells the protein is localized to the apical membrane, where it may participate in excretion of certain compounds from the body *(31)*. Sequence homology between P-glycoprotein and CFTR is highest in the nucleotide-binding folds, and considerable similarity exists in their predicted structural motifs. The two proteins share a large internal repeat, and each has 12 transmembrane helices, 2 ATP-binding sites in similar positions, and cytosolic loci for both the N and C termini. A major difference is that the two halves of the CFTR protein are linked by a large domain, coded by a single 723 base-pair exon; the P-glycoprotein lacks this region.

Transport proteins of the P-glycoprotein family bind ATP and are probably ATPases *(32)*. Loss of a single ATP-binding site from the P-glycoprotein affects its ability to confer drug-resistance. This may be relevant because the protein defect found in the majority of CF individuals is a deletion of a single phenylalanine in the first nucleotide-binding fold, which may interfere with the ability of the CFTR to utilize ATP.

How can this new information be related to existing information on ion transport defects and the higher-order problems that derive from them? To put the CFTR in perspective, we need to consider some general properties of ion-transporting epithelia and some selected accounts of how transport is altered in cystic fibrosis.

### *Basic Mechanisms of Secretion and Absorption*

All cells have mechanisms for moving ions across their plasma membranes to help regulate their internal environment. Phospholipid bilayers are relatively permeable to water and small nonpolar molecules but are a billion times less permeable to small charged ions like $Na^+$ and $Cl^-$. To control the movements of such ions, cell membranes contain several classes of transmembrane proteins to facilitate the movement of ions. Conceptually, the simplest of these trans-

membrane proteins are the ion channels, which literally provide an open pore for ions to flow through the membrane. Channels have transport rates in the range of $10^7$ ions/s. Ion carriers or exchangers go through a conformational change each time an ion is transported and are therefore limited to maximal transport rates of about $10^5$ ions/s, whereas ion-transporting ATPases, which use the energy of ATP hydrolysis to move ions against their concentration gradient, are much slower and are limited to transport rates of about $10^3$ ions/s or less. Given the great variety of ion-transporting molecules, it is interesting that cells have never devised a way of actively transporting water directly. Instead, all movement of water across cell membranes occurs indirectly, by osmosis, following the direct movement of ions.

Although every cell has some means for transporting ions, fluid-transporting epithelial cells, which provide the crucial operating portions of all exocrine glands, have evolved several specializations that give them the unique ability to move water vectorially. Transporting epithelial cells organize themselves into sheets, often one cell thick (monolayers), and their special properties only fully emerge after they form such tissues (Fig. 3).

A key specialization that makes possible the organization of transporting epithelial sheets is the tight junction *(33)*. Tight junctions form a barrier that limits diffusion between adjacent cells; this diffusional pathway is called the paracellular pathway. Tight junctions also form a barrier that limits lateral diffusion within the outer leaflet of the plasma membrane. Thus, transmembrane proteins that are inserted on one side of the tight junction stay there; this and other mechanisms make possible the polarization of epithelial cells into apical and basolateral membranes. The distribution of ion-transporting proteins in the apical and basolateral membranes varies according to the job the epithelium has to do. A great many transport processes are powered indirectly by $Na^+$, $K^+$-ATPase, which is almost always localized in the basolateral membrane of epithelial cells, and may be present at densities as high as 10,000 molecules/$\mu m^2$ *(34)*. The $Na^+$, $K^+$-ATPase keeps $Na^+$ levels inside the cell low and $K^+$ levels high. The large electrochemical gradient for $Na^+$ is an energy source that the cell can use to power other kinds of ion transport.

Fluid secretion is controlled by hormones and neurotransmitters that usually, but not always, bind to receptors on the basolateral membrane. An important generalization is that *all exocrine cells have*

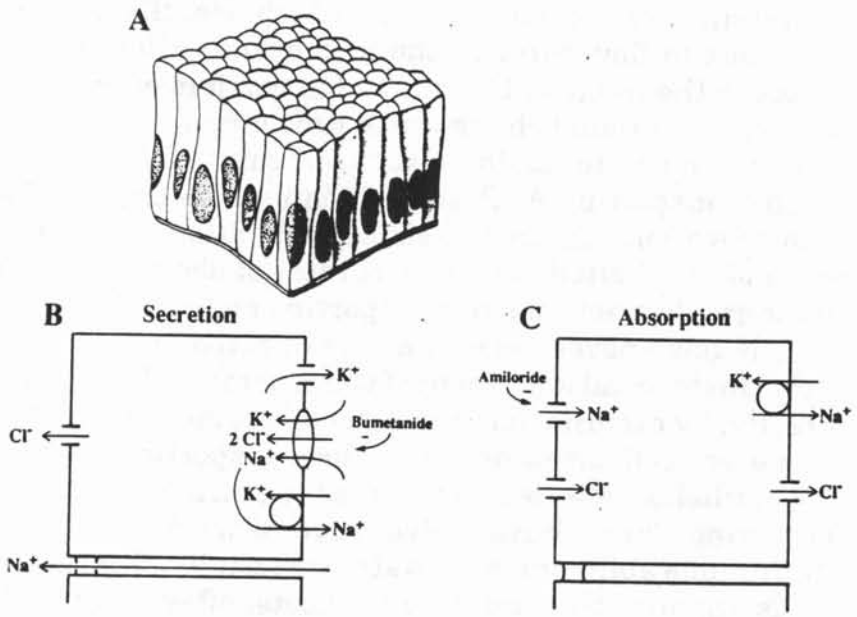


Fig. 3. Ion-transporting epithelia. **A.** Semischematic diagram of a layer of columnar epithelial cells: Basement membrane at bottom, apical membrane surface at top. **B** and **C.** Schematic, cross-sectional view of major elements involved in the transport process. Cells are connected by tight junctions that can be either permable to some ions ("leaky epithelia," illustrated at left) or impermeable to ions ("tight epithelia," shown at right). Basolateral membranes have $Na^+$, $K^+$-ATPase and may have transporters for increasing the intracellular Cl– concentration. Apical membranes may have channels to Cl–, $Na^+$, or both. **B.** A Cl– secreting epithelium: Cl– is moved uphill into the cell by coupling to a bumetanide-sensitive $Na^+$, $K^+$, 2 Cl– cotransporter, and then flows out across the apical membrane via Cl– channels, whereas $Na^+$ flows via the paracellular pathway, and water moves mainly transcellularly. **C.** A $Na^+$ and Cl– absorbing epithelium. Here the driving force is provided by $Na^+$ entry into the cell and its subsequent extrusion by $Na^+$, $K^+$-ATPase, with passive, transcellular Cl–movement. In this kind of epithelium the tight junctions are impermeable to ions and the entire epithelium can have a relatively low permeability to water.

*nonexcitable membranes*. This means that the action of transmembrane proteins in different parts of the cell must be coordinated by intracellular messengers such as cAMP and $Ca^{2+}$. The hallmark of fluid-secreting epithelia is that they produce large net movements of ions with little change in the transmembrane voltage.

A general model of $Cl^-$-mediated secretion has guided much research in epithelial transport physiology *(35)*. In this model the flow of $Cl^-$ is out of the cell and into the lumen with $Na^+$ following via a

leaky, paracellular pathway (Fig. 3B). The model requires the cell to increase the intracellular concentration of $Cl^-$ above its electrochemical equilibrium so that when the apical Cl– channels open, Cl– exits the cell. This is achieved by having a special Cl– transporter in the basolateral membrane that uses the $Na^+$ gradient to move Cl– into the cell, uphill against its electrochemical equilibrium. This general model of Cl– secretion has been established with varying degrees of rigor in many systems, including human airway epithelia *(36)* and the intestine *(37)*.

A general model of salt absorption, originally developed by Ussing and his collaborators for frog skin, has also proven to be applicable to a great many epithelia (Fig. 3C). As with Cl– secretion, the main source of energy for NaCl absorption is $Na^+$, $K^+$-ATPase, which keeps the cytosolic levels of $Na^+$ low, creating a favorable electrochemical gradient for $Na^+$ entry across the apical membrane. $Na^+$ entry occurs via a specific type of channel that is blocked by micromolar concentrations of the diuretic amiloride. The $Na^+$ flux across the epithelium creates a negative potential at the apical membrane surface so that anions, typically $Cl^-$, are also absorbed. Pathways for $Cl^-$ absorption appear to differ in various epithelia: In sweat ducts, most $Cl^-$ moves transcellularly *(38)*, whereas in airway epithelia, the major pathway is paracellular *(39)*.

Because $Na^+$ entry is the rate-limiting step for salt absorption, the amiloride-blockable $Na^+$ channels are heavily regulated. One particularly interesting example of regulation is affected by the external $Na^+$ concentration itself: The $Na^+$ permeability varies inversely with the external $Na^+$ concentration, being reduced to half maximal with $Na^+$ concentrations of 7–45 m*M*. Note that this is a negative feedback mechanism that helps maintain a relatively constant influx of $Na^+$ into the cells over a broad range of mucosal $Na^+$ concentrations.

Sodium "self-inhibition" was recently investigated with single-channel techniques *(40)*. Cell-attached recordings were made on amphibian kidney cells (A6 cell line) with a pipet containing high $Na^+$ (129 m*M* NaCl). After amiloride-blockable $Na^+$ channels were recorded, the bath $Na^+$ concentration was lowered from 129 to 3 m*M*, although the pipet solution remained unchanged. Within minutes, the open channel probability of the $Na^+$ channels within the patch increased and the minimum number of such channels (as judged by simultaneous openings) also increased. Increased channel openings

also occurred when amiloride was added to the bath solution. Since in either case the manipulations could not have directly affected the membrane inside the pipet tip, reduced $Na^+$ influx presumably increased channel open probability via an intracellular regulatory pathway. Protein kinase C may be involved, since inhibitors of protein kinase C also increased $Na^+$ channel activity, whereas activators of the enzyme reversed the effects of low external $Na^+$.

## *Ion Transport Defects in Cystic Fibrosis: Chloride and Sodium*

The major ion transport defect in CF is a drastic reduction of $Cl^-$ permeability *(18)*. Reduced $Cl^-$ permeability is found both in absorptive cells and secretory cells and is detectable in unstimulated cells. However, for most secretory cells, the defect is seen most clearly when cells are stimulated to secrete $Cl^-$—the secretory response appears to be almost totally abolished in sweat secretory *(23)*, airway *(41)*, and intestinal *(3–5)* cells.

It is not yet known whether this is the only fundamental defect in CF. An increase in absorption of $Na^+$ has also been shown for cultured nasal epithelia from CF subjects *(42)*. Increased $Na^+$ transport is apparently caused by increased apical $Na^+$ permeability, but it is not clear whether that increase is a direct result of altered regulation by the CFTR or is an indirect consequence of the reduced $Cl^-$ permeability. Experiments to decide the issue would be easier if a specific and effective blocker was available for the apical $Cl^-$ channel. It is interesting that when canine airway epithelia were bathed in a $Cl^-$ free medium, agents that elevate cAMP caused an increase in $Na^+$ absorption *(43)*. It has also been shown that diphenylamine-2-carboxylate (DPC), which blocks $Cl^-$ channels in many epithelia, stimulates $Na^+$ transport in frog skin. This effect was seen even in $Cl^-$ -free Ringer *(44)*. How might increased $Na^+$ permeability occur? One possibility is that the normal down-regulation of $Na^+$ permeability in response to external $Na^+$ is defective in CF cells, leading to chronically elevated $Na^+$ transport. If true, it would be predicted that amiloride-blockable $Na^+$ permeability of affected epithelial cells from CF subjects should approach normal values at low external $Na^+$ concentrations, but should fail to show self-inhibition as apical levels of $Na^+$ were increased, leading to higher than normal $Na^+$ conductances at

these upper levels. Such experiments have not been done to my knowledge, but even if successful they would not answer the question of whether the aberrant regulation was a secondary or primary defect. Thus, we should keep an open mind about the possibility of a direct involvement of $Na^+$ permeability in CF.

## Chloride Impermeability in CF Tissues

The remainder of this review focuses on $Cl^-$ impermeability and its relation to the CFTR. Ion transport defects in CF have been seen most clearly in the sweat gland, which, because of its relative accessibility and freedom from infection or damage caused by impacted mucus, has played a key role in CF research. An overview of sweat gland function is presented in Fig. 4 *(45,46)*. The coiled portion of the sweat gland moves ions, mainly $Na^+$ and Cl–, across a single layer of cells into the lumen. These ions are followed by water to produce a fluid called primary sweat that is similar to an ultrafiltrate of blood. Secretion builds up hydrostatic pressure, which is resisted by a network of myoepithelial cells that surrounds the coil to provide mechanical support—thus, the sweat is forced into the duct and eventually to the surface of the skin.

Thermally induced sweat secretion occurs when muscarinic receptors on the secretory cells are stimulated with acetylcholine released from autonomic neurons. Reflex sweating can be completely blocked with the muscarinic antagonist atropine, and sweating produced by direct infusion of cholinergic agonists can be blocked by removing external sources of $Ca^{2+}$, suggesting a role for $Ca^{2+}$ as an intracellular messenger for thermal-reflex sweat secretion *(45,46)*. The production of primary sweat in response to thermal challenge or infused acetylcholine appears to be completely normal in people with CF (Fig. 5B).

Sweating can also be produced with a variety of agents, like isoproterenol, that bind to β-adrenergic receptors. Sweating to β-adrenergic agonists is *completely absent in people with CF and is reduced to half normal in CF heterozygotes* (Fig. 5, *23,47*). Although the physiological relevance of the β- adrenergic sweat pathway is unknown, it remains to this day the only unequivocal example in which fluid secretion has been shown to be affected in CF, and it is of special significance since a similar, but more physiologically relevant, reduction

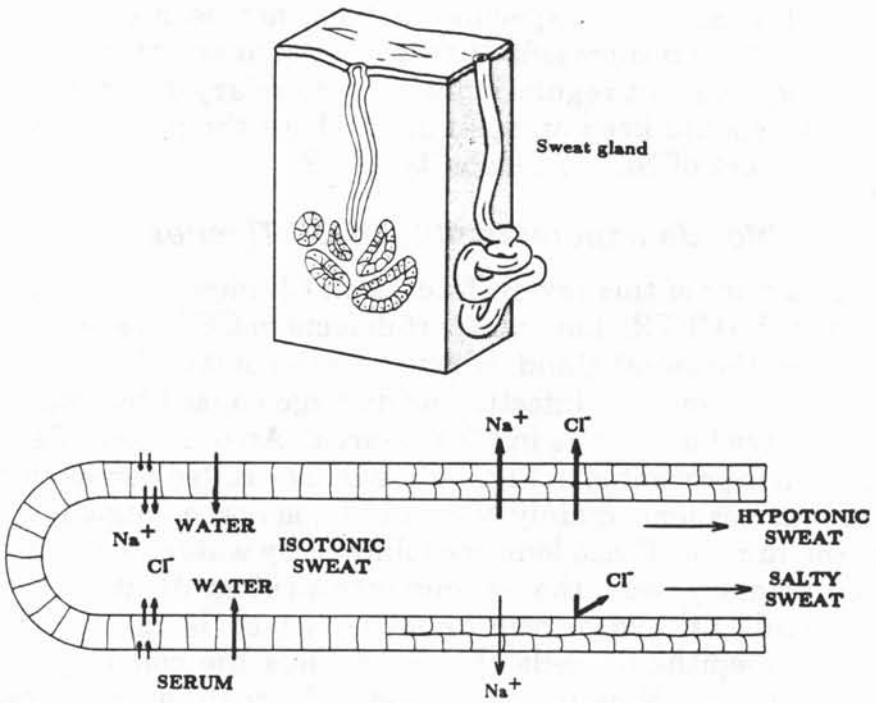


Fig. 4. Overview of sweat gland function and defects found in CF. **A.** A semi-schematic diagram of a sweat gland *in situ*. **B.** Full schematic of a gland. The secretory portion (single cell layer) secretes a nearly isotonic fluid, probably using the mechanism outlined in Fig. 3B. The reabsorptive duct (double cell layer) removes as much as 80–90% of the salt from the sweat, using, at least in part, the mechanism outlined in Fig. 3C. In CF, the $Cl^-$ permeability of the duct is reduced drastically, which directly prevents the absorption of $Cl^-$ and indirectly prevents the absorption of $Na^+$ (via the large, lumen negative electrochemical potential that develops across the duct epithelium). The characteristically salty sweat of CF patients results.

of fluid production might well explain the pathologies in intestine, pancreas, cervix, vas deferens, and lung, since in each of these organs apical $Cl^-$ efflux is thought to be a prerequisite for fluid secreton.

Among the consequences of β-adrenergic stimulation is the activation of adenylate cyclase and elevation of cAMP levels within the cell, leading to phosphorylation of many proteins via cAMP-dependent protein kinase. These pathways have been the focus of intense interest by CF researchers, but β-adrenergic stimulation has other effects that, though less well understood at present, may nevertheless be important for regulating secretion in some systems. Of great potential interest is the recent report that excessive fluid secretion

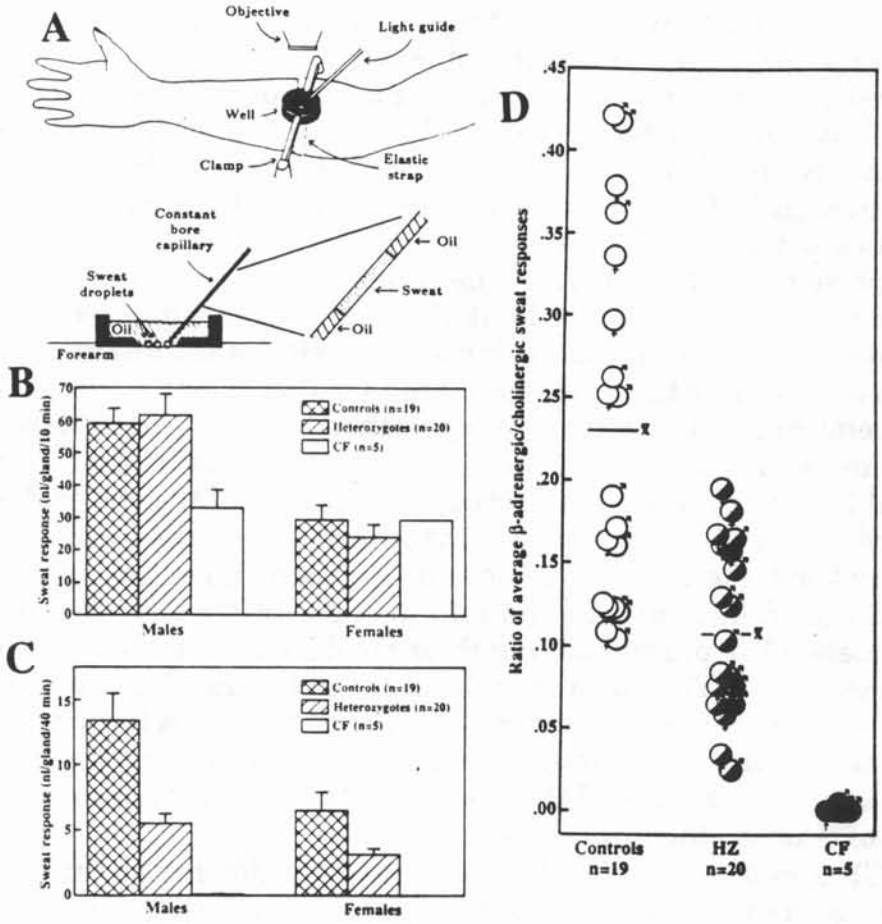


Fig. 5. Sweat secretory defects in cystic fibrosis. The human eccrine sweat gland can be stimulated to secrete with either cholinergic or β-adrenergic agents; secretion to the former is normal in CF, secretion to the latter is totally abolished in CF homozygotes and is half normal in heterozygotes. **A.** Testing method: After an intradermal injection of agonists, sweat droplets from individual glands are collected at frequent intervals under oil in constant bore microcapillaries and measured microscopically. **B.** Sweat responses to cholinergic stimulation are normal (the lower rate for CF males was not significant and probably represented the effects of inactivity). **C.** Sweat responses to β-adrenergic stimulation are abolished in CF subjects and reduced to half normal in heterozygotes. **D.** When the ratio of β-adrenergic to cholinergic responses is plotted for each subject, a control population shows wide variability, and the sex difference disappears; heterozygotes show decreased variability and a mean about half that of normal subjects, and CF subjects show no response. Thus the CFTR appears to be rate-limiting for β-adrenergic stimulated sweating, with a single CFTR serving as a functional unit *(47)*.

induced in the intestine by cholera toxin, which has long been thought to be exclusively a function of excess cAMP production, may instead be mediated largely by prostaglandins *(48)*.

The secretory defect in CF sweating is only detectable in the laboratory, but a major defect in salt reabsorption from the sweat is apparent to anyone with CF and is at present the most frequently used diagnostic sign for CF. We now understand how the high salt content of sweat comes about *(18)*. As primary sweat moves along a normal reabsorptive duct, the ductal epithelium reabsorbs up to 85% of the NaCl from the ductal fluid without absorbing much water, resulting in considerable dilution of the salt concentration of the sweat. Reabsorption is driven by the large electrochemical gradient for $Na^+$. The apical membrane of the duct has a high density of amiloride-blockable $Na^+$ channels, through which $Na^+$ flows into the ductal cells. The basolateral $Na^+$, $K^+$-ATPase then transports $Na^+$ out of the cell and into the blood. This $Na^+$ movement instantaneously creates a negative voltage in the lumen, that provides an electrochemical gradient that forces $Cl^-$ out of the lumen and into the ductal cells via apically located $Cl^-$ channels. (The paracellular pathway in duct cells has a high resistance to ion flow.) The apical membrane of sweat ducts must contain either a large number of $Cl^-$ channels or channels with high conductance, because the conductance of a normal sweat duct is among the highest known for epithelial tissues (about 110 $mS/cm^2$) and the major portion of this conductance is to $Cl^-$ *(21)*.

In CF sweat ducts, the $Cl^-$ conductance of the epithelium is reduced to a virtually undetectable level, and the duct behaves as though it were permeable only to $Na^+$. Thus, when $Na^+$ attempts to flow out of a CF duct unaccompanied by Cl–, it creates a large excess of negative charge in the duct that sets up an opposing electrical gradient for $Na^+$ and so greatly retards its movement. The net result is that both $Na^+$ and Cl– are poorly reabsorbed by the CF duct, leading to the high salt content of CF sweat.

How can the defect in the CFTR protein explain both the lack of secretion to β-adrenergic agonists and the greatly reduced $Cl^-$ permeability of the duct? Since all examples of CF are caused by mutations in a single gene, and since almost half of the people with CF are homozygous for the $\Delta F_{508}$ allele, it is clear that the severely reduced $Cl^-$ permeability of the sweat duct and the failure of β-adrenergic sweating have the same fundamental cause. A salient possibility is that a

specific type of $Cl^-$ channel has been rendered nonfunctional by the mutation. By analogy with many other tissues, it is possible that sweating in response to β-adrenergic agonists involves an increase in apical $Cl^-$ conductance of the secretory cells and that this increase does not occur in CF sweat secretory cells.

Epithelial $Cl^-$ secretion can be measured in Ussing chambers. Such measurements have shown that the human airways *(49,50)* and intestine *(3–5)* can be induced to secrete $Cl^-$ and that in each case stimulated $Cl^-$ secretion is absent in CF tissues. The intestinal examples are particularly interesting because defective secretion in CF has been demonstrated for pathways employing cAMP, cGMP, and $Ca^{2+}$, which is strong evidence that the defect occurs after the point of convergence of these diverse, stimulus-secretion pathways.

Thus, both the pathophysiology and basic studies of transport physiology suggest that a defect in $Cl^-$ permeability is the basic physiological alteration in CF. It has not yet been established that the same $CF^-$ vulnerable $Cl^-$ pathway is used for both absorption and secretion, but the evidence is conclusive that some cells are able to transport $Cl^-$ in spite of the CF mutation, either because they possess alternate pathways for moving $Cl^-$, or alternate ways of activating the CF-vul-nerable pathway that are spared by the CF mutation. So the question is now put into sharp focus: What is the relation between the CFTR and the CF-vulnerable $Cl^-$ permeability pathway?

## *Single-Channel Studies of the Transport Defect: A Candidate "CF Channel"*

The evidence from the sweat gland, viewed in the context of the general model of $Cl^-$ secretion, led quickly to studies of $Cl^-$ secretion in cultured human cells. These were first successful with human airway epithelia, where it was shown that stimulated $Cl^-$ secretion could be maintained in cultured monolayers of normal human airway cells, but that such secretion was virtually absent in monolayers from CF individuals *(41,49)*. This led immediately to a search for the ion-channel basis of the defect. Two groups quickly identified a distinctive, large-conductance, outwardly rectifying $Cl^-$ channel in cultured airway cells that appeared to be activated by a cAMP-dependent phosphorylation event in canine cells and in normal human, but not CF, cells *(50–52)*. A striking extension of the work occurred when it was

shown that the channel from normal, but not CF, cells could be activated when tiny patches of cell membrane were excised from the cell and exposed to the catalytic subunit of cAMP-dependent protein kinase + ATP, neither reagent alone being sufficient *(53,54)*. This localized the defect to the patch of excised membrane and strongly linked the defect to a phosphorylation event or its immediate consequences. These reports, subsequently verified in other laboratories *(55,56)* and extended to lymphoblasts *(57)*, appear to identify the channel involved in CF. However, the experimental procedure involved is difficult, and several peculiar properties of the channel being studied have introduced uncertainty about the relationship of this channel to the CF defect.

The technique used in these experiments, termed patch-clamp recording *(58)*, allows a tiny patch of the cell membrane to be sealed into the tip of a glass pipet (Fig. 6). In favorable circurcumstances the electrical resistance of this glass-membrane seal is 10–100 gigaohms ($10^{10}$–$10^{11}$ ohms), allowing the transmembrane voltage across the patch to be set (clamped) to any voltage the experimenter desires. Ion currents as small as a fraction of a picoampere ($10^{-12}$ amperes) can then be recorded. The area of membrane in the patch can vary from 1–10 $\mu m^2$ depending upon the size of the pipet tip and experimental conditions *(59)*. Patches of this size often have just one or a few channels in them, permitting detailed studies of channel behavior. In the cell-attached mode, a seal is made on an intact cell, which can then be stimulated while observing any changes in the probability of finding channels open. In the excised patch method, the mechanical bond between pipet and membrane is usually sufficiently strong to allow the membrane patch to be pulled from the cell, exposing the cytoplasmic side of the patch membrane to the bath solution. This is of great aid in the study of channels that are regulated by cytosolic factors.

The patch-clamp technique has been applied to the CF problem in several cell types with variable results. In cultured dog tracheal cells, which are a classic preparation for studying $Cl^-$ secretion, the rectifying $Cl^-$ channel was found in only 2–10% of patches, and only about 1/3 of all attempts to activate the channels with isoproterenol were successful *(50)*. In contrast, in the Jurkat T lymphocyte cell line and in several B lymphoblast lines, an apparently identical channel was found in 76% of all patches tested and was almost invariably activated by a variety of agents in normal, but not CF, cells *(57)*.

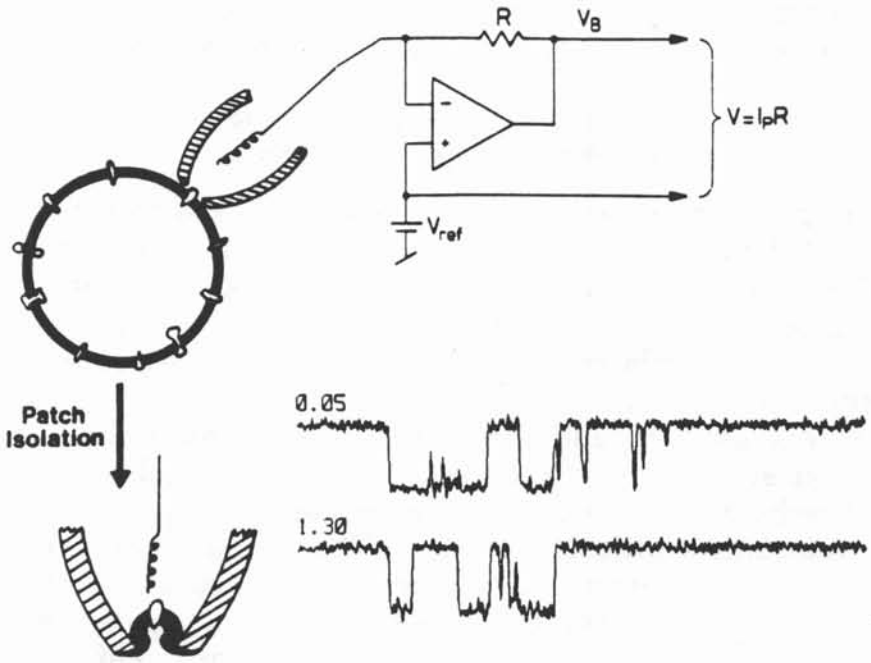


Fig. 6. Overview of patch-clamp recording (not to scale). Schematic shows a cell with transmembrane proteins, against which the tip of a polished glass pipet is placed. With gentle suction, some of the membrane is pulled into the tip, where it seals tightly, allowing currents in the range of $10^{-12}$ amperes (picoamperes) to be recorded. In this configuration (the "cell-attached" mode), the operation of the channel is most normal. The patch can also be excised from the cell, and its cytoplasmic surface exposed to solutions of controlled composition. Records show a typical feature of many channels—they switch rapidly from full-conducting to nonconducting states, producing rectangular currents *(adapted from 59)*.

In an attempt to try to understand this variability and perhaps bring it under experimental control, we examined both epithelial cells (dog and human sweat gland and airway cells) and lymphoblast cell lines (*60–65* and unpublished results). Unfortunately, although our own experiments with both types of cell have yielded consistent results, the results were unexpected: We have found no evidence to support a major role for the rectifying $Cl^-$ channel in agonist-mediated increases in $Cl^-$ conductance in either epithelial cells or in lymphoblasts. Similar failures have recently been reported for cultured sweat gland cells *(66)* and for fetal pancreatic cells *(67)*, where the rectifying $Cl^-$ channel was detected in only 5 of 194 excised patches and in only a single cell-attached patch. The work on human pancre-

atic cells is particularly interesting, because an alternate $Cl^-$ channel has been proposed to mediate $Cl^-$ secretion in those cells.

### *Reservations About the Role of the Rectifying $Cl^-$ Channel*

How might such differences among laboratories come about? The properties of the rectifying $Cl^-$ channel certainly make it difficult to study. The apparent channel density is highly sensitive to the testing method, since there is often no spontaneous cell-attached activity in either resting or stimulated cells, yet the channel may open spontaneously when the patch is excised, and this propensity to open after excision is increased by depolarization and the duration the patch is held after excision. Depolarization-induction of channel activity has no clear threshold, has a long and variable latency, may be influenced by $Ca^{2+}$ levels, and is difficult to reverse. The complexities of voltage-induction of channel activity are, if anything, exceeded by the reported properties of agonist or kinase gating: The latency of each is very long and variable (up to 8 min), and the proportion of channels responsive to agonist is highly variable, ranging from near zero in some studies to near 100% in others. To date, there is no convincing evidence that gating is reversible. Given these properties, it is not surprising that dose-response relationships have not been determined. In sum, the evidence that this channel can be activated in cell-attached patches by application of agonists must still be considered circumstantial.

The difficulties inherent in cell-attached studies of the large, rectifying $Cl^-$ channel, plus evidence that the large, rectifying $Cl^-$ channel is only infrequently active when cell-attached, may help explain why so few cell-attached experiments have been published, and why experiments with the more easily studied excised patches now predominate. Although excised patches offer many experimental advantages, the channels in such recordings are no longer able to interact with cytosolic and cytoskeletal elements that might be important for their normal function. Nevertheless, in the majority of studies of excised patches published to date, consistent and striking differences have been reported for responses of channels from normal and CF cells to exposure to catalytic subunits of cAMP-dependent protein

kinase (PKA) and protein kinase C (PKC, refs. *53–57, 68*). The results indicate that both PKA and PKC can activate channels in normal, but not CF, cells. The data as shown appear convincing. However, there have been frequent failures to activate this channel by almost everyone who has investigated it (personal communications). Since these failures have largely been attributed to inadequate technique, inactive reagents, or unidentified aspects of culture conditions, they usually have not been reported. Thus, the actual success rate for activating this channel is lower, and perhaps much lower, than the published reports indicate. In addition to experimental difficulties, the evidence that the rectifying $Cl^-$ channel has a special role to play in the CF-defective $Cl^-$ pathway is somewhat weakened by the tissue distribution of this channel, which does not appear to be an exclusive component of apical membranes of transporting epithelia, but is also found, at levels equal to or exceeding those of epithelial tissues, in fibroblasts *(69)*, lymphoblasts *(57)*, and keratinocytes *(5)*. Recent attempts to detect mRNA transcripts of the CFTR protein in lymphoblast or fibroblast cell lines were unsuccessful *(25)*. However, this is again negative evidence, and the issue will not be settled until expression studies are completed. We are thus left in a quandary: The rectifying $Cl^-$ channel may be identical with the CFTR protein or be its accomplice, but it is also possible that it is an innocent, but conspicuous, bystander.

### *Experiments with $Ca^{2+}$ and PKC*

It is a striking finding that sweating produced by cholinergic stimulation appears to be unaffected by the CF mutation, whereas β-adrenergic-meditated sweating is completely lost. If a $Ca^{2+}$-mediated $Cl^-$ secretory pathway were spared in CF, it might be possible to amplify it pharmacologically and restore at least partial function. This issue is thus of great interest, but so far the results are quite confusing. Since it is known that cholinergic stimulation requires extracellular $Ca^{2+}$ and induces a rise in cytosolic $Ca^{2+}$, one possibility is that $Ca^{2+}$ directly activates the "CF" $Cl^-$ channel, and this action is retained in CF tissues. Evidence to that effect has been obtained in some experiments with cultured airway cells *(52)*, but not others *(51)*. A less direct effect of $Ca^{2+}$ is possible, since several studies have shown

that addition of the $Ca^{2+}$ ionophore A23187 appears to stimulate $Cl^-$ secretion in monolayers of cultured CF airway cells *(49, 70)*. Interestingly, this effect is not general, since $Cl^-$ secretion to all forms of stimulation, including $Ca^{2+}$ -elevating agents and $Ca^{2+}$ ionophores, is lost in CF intestinal tissues *(3–5)*. In addition, we have been unable to restore normal $Cl^-$ permeability to CF sweat ducts with any $Ca^{2+}$-elevating agent we have tried, including A23187 (Wine and Lewiston, unpublished results).

The complexities of $Ca^{2+}$ regulation of rectifying $Cl^-$ channels in airway epithelia are well-illustrated by recent experiments in which the catalytic subunit of PKC was added to excised patches. At low levels of $Ca^{2+}$ (about 100–200 n*M*), PKC addition activated channels in patches excised from normal, but not CF, cells *(53, 54)*. At higher levels (about 1 μ*M* and perhaps lower), PKC caused inactivation of the channel, and this property was retained in excised patches from CF cells *(54)*.

### Are There Alternatives to the Rectifying $Cl^-$ Channel?

There is no doubt that $Cl^-$ permeability is drastically reduced in many CF tissues. In spite of the issues raised above, the rectifying $Cl^-$ channel remains the leading candidate for involvement in the CF defect. The acknowledged complexity of this channel's behavior cuts both ways—it could lead to spurious results, but could also explain why real differences between CF and normal tissues have not been detected by all investigators. Until recently, no other candidate channels had been identified. (A 20 pS linear $Cl^-$ channel, identified in initial work, has not been pursued.) That may be changing. A different kind of regulated, apical membrane $Cl^-$ channel has been detected in cultured cells from fetal human pancreas. This channel is a 4–7 pS linear channel that is spontaneously active in unstimulated cells, but shows a threefold increase in activity when cells are stimulated by secretin or by forskolin + 8-Br-cAMP. Stimulation is reversed when the agonist is removed *(67)*. It may be important that this channel becomes less active when excised from the cell, suggesting the requirement for a factor present in the cytosol but not the typical bathing solution. Because of the small size of this channel and the rapid cessation of its activity in excised patches, it might have been missed in earlier studies of other epithelia.

## Concluding Remarks

Basic physiological studies of CF have provided a wealth of information on the basic defect, but further progress would have been difficult without the tremendous boost provided by identification of the gene. With luck, this should be the last review of CF that is forced to speculate about the nature of the basic physiological defect. The following hypotheses can soon be definitively tested (Fig. 7).

### *Is the CFTR Protein a $Cl^-$ Channel?*

This is perhaps the simplest hypothesis, consistent with much data, and now easy to test. The CFTR protein can be overexpressed in cells having either minimal or well-characterized $Cl^-$ conductances, and the membrane assessed with electrophysiological techniques, including patch-clamp recording. If it is a channel, it may not be the one that has received the most attention.

### *If the CFTR Protein Is a $Cl^-$ Channel, Is That all It Is?*

The $C^-$ impermeability hypothesis can readily explain excessive salt in the sweat and failure of β-adrenergic sweating, and is consistent with dehydrated cervical mucus and meconium ileus. With only slight elaboration it can account for the pathologies observed in pancreas, vas deferens, and salivary glands, since in all of those organs there is some evidence that fluid secretion is a $Cl^-$-dependent process. But how does one go from altered $Cl^-$ conductance to lung infection?

Impairment of mucociliary clearance may contribute to CF lung infections *(71)*, since decreased $Cl^-$ secretion might deplete the fluid content and increase the viscosity of the 10μm-thick film of fluid and mucus that is thought to be a crucial component of lung mucosal defenses (e.g.,*72*). However, in view of the inconsistent evidence for reduced mucosal clearance by CF lungs *(73)* and the different clinical profile presented by patients with immotile ciliary syndrome *(74,75)*, we should remain alert to other possibilities. In particular, the colonization by *Pseudomonas* and the conversion of *Pseudomonas* to a mucoid (alginate-producing) form is common in CF and rare in other kinds of chronic lung diseases (Fig. 8). When mucoid strains of *Pseudomonas* are isolated from CF lungs and placed in culture, they rapidly revert to the nonmucoid form *(76)*, suggesting the presence of

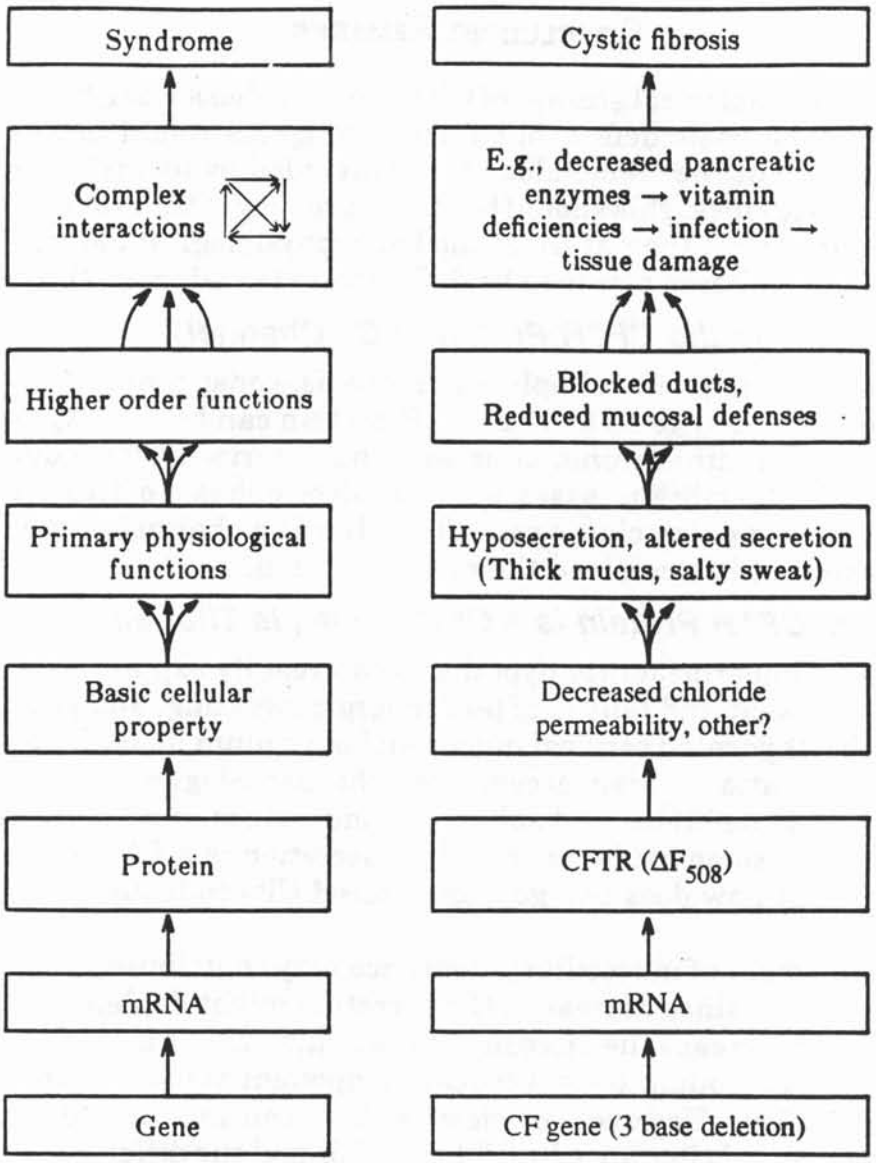


Fig. 7. Summary of pathways from gene to syndrome emphasizing the $Cl^-$ hypothesis. Column on left shows a general scheme for any single gene disease. It is recognized that complications may arise at very low levels: For example, alternative splicing may allow a single gene to produce several mRNAs, cell-specific posttranslational modification can further differentiate the protein products of a single gene, and several basic cellular properties could be affected by a defect in a single protein. Column on right shows the extent to which the steps in the pathway from gene to syndrome have been filled in.

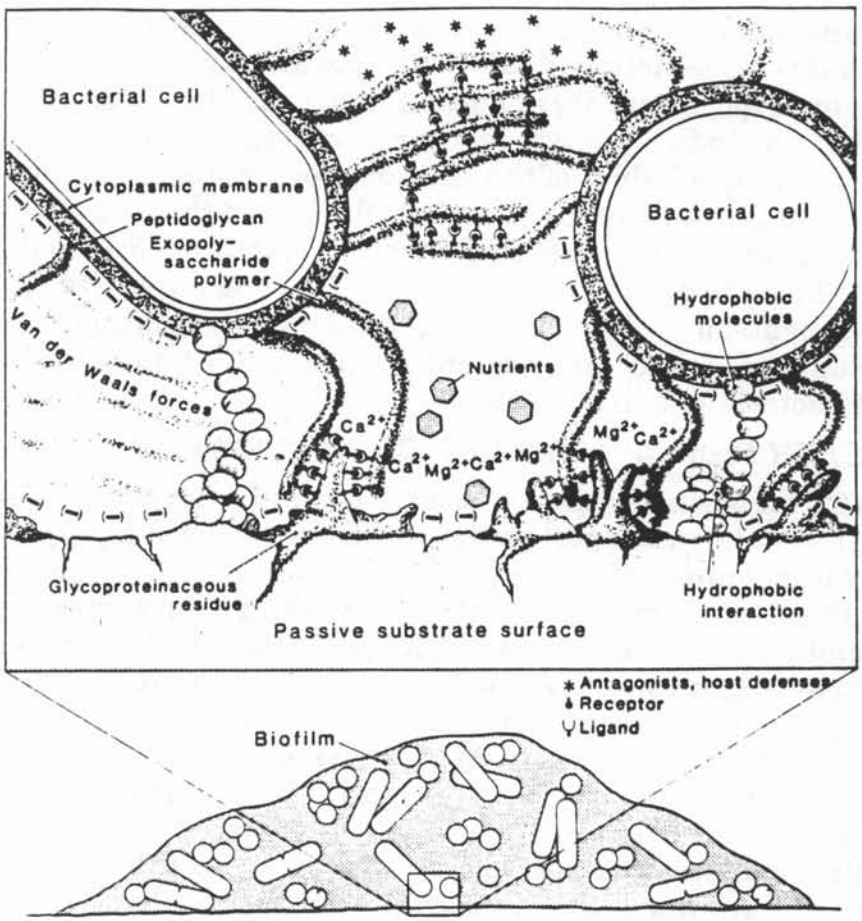


Fig. 8. Bacterial adherence and the formation of alginate biofilms. Shown here is hypothetical mechanism of bacterial adherence in osteomyelitis, with secondary formation of mucoid encapsulation. The CF lung may be altered in at least two ways: Protection against bacterial adherence appears to be compromised and formation of alginate biofilm appears to be more likely, leading to the type of colonization shown here (reproduced with permission from ref. *80*).

transforming conditions in the CF lung. Great strides in understanding the control of *Pseudomonas* alginate synthesis have been made by Chakrabarty and his colleagues, who have used molecular-genetic techniques to identify many of the genes responsible for alginate production in *Pseudomonas (2)*. Of special interest is the identification of key components in a regulatory pathway for alginate synthesis and evidence that the alginate synthesis pathways are activated in cer-

tain strains of *Pseudomonas* by exposure to increased osmolarity *(2)*. Although it is extremely difficult to sample airway secretions, some evidence for hypoosmolarity of normal lung fluids has been obtained *(77)*, which may indicate active reabsorption of salt in excess of water. By extrapolation, a failure of the reabsorption process in CF airways might lead to excessively salty airways fluids and thus create conditions favorable for alginate production by resident *Pseudomonas* strains. Thus, the path to infection in the CF lung may have at least two components; an inital defect in the lung's defenses against bacterial colonization, and a subsequent predisposition for transforming resident bacteria to a mucoid form.

### *If the CFTR Protein Is Not a $Cl^-$ Channel, What Might It Be?*

The CFTR has a complex structure. Its similarity to family of related ATP-binding transport proteins, including the P-glycoprotein, is deeply intriguing and has fueled speculation that the CFTR is an ATPase that transports something, perhaps something large, across some membrane. However, the CFTR also shows tantalizing modifications that might confer channel properties. The most consistent physiological evidence makes it clear that the CFTR must, at a minimum, regulate $Cl^-$ permeability. It is not evident how it might do that if it is not a channel, nor whether that is all it does. Perhaps the CFTR is a multifunctional protein. Given its large size and the evidence that multiple alleles give rise to cystic fibrosis, it is interesting that there are no variants of CF in which the CF gene suffers deletion of a large region so far. Large deletions might be expected in a gene of this size; their absence in the CF population could mean that they are highly lethal, suggesting that the CFTR protein retains a crucial function in CF individuals. (Depending on how one assigns functions, this possibility is not inconsistent with the idea that more than one function is lost in most types of CF. Control of $Cl^-$ permeability may just be one of a family of processes controlled by the CFTR, one branch of which is impaired in CF.)

It is risky, but probably obligatory, to conclude a review of this kind by speculating on the possibilities for ameliorating or curing CF. Beyond the obvious statement that the discovery of the gene will cause an explosion of new understanding, there may be additional hope in the complexity of the protein and the subtlety of its defects. If it turns out that the CFTR protein does retain crucial functions in

CF individuals, it might leave hope for a way to tease out some residuum of the lost function(s). In at least one system, the β-adrenergically-stimulated eccrine sweat gland, secretion appears to be rate-limited by the CFTR, such that heterozygotes, who presumably have a 50/50 mixture of normal and mutant CFTR protein, secrete half as much as normal *(47)*, yet have none of the life-threatening aspects of CF.* In systems like the pancreas, retention of as little as 1–2% of normal volumes of pancreatic juice enable digestion to proceed normally *(3–5)*, and similar safety margins appear to be the rule in other organ systems. Thus , a very small patch might go a very long way toward enabling people with CF to lead normal lives.

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*Incidentally, this would seem to rule out schemes in which the CFTR functions as a dimer or higher order structure.

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