tilled water. The numbers of surviving spores and His^+ revertants were measured as described in (*3*).

The induced mutation frequency observed in irradiated spores was decreased by the heat treatment in parallel with the increase of spore survival (Fig. 1). The relation between the mutagenic damage and the lethal damage is seen more explicitly when the induced mutation frequency is plotted against the logarithm of the surviving fraction (Fig. 2). The ratio of the induced mutation frequency to the surviving fraction is unchanged whether the irradiated spores were given a heat treatment or not. This unchanged ratio indicates that DNA damage is the origin of the thermorestoration of the lethal damage as well as of the mutagenic damage.

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References and Notes

1. E. L. Powers, R. B. Webb, C. F. Ehret, *Radiat. Res. Suppl.* **2**, 94 (1960); R. B. Webb, E. L. Powers, C. F. Ehret, *ibid.* **12**, 682 (1960).
2. H. Tanooka and F. Hutchinson, *Radiat. Res.* **24**, 43 (1965).
3. H. Tanooka, *Mutat. Res.* **42**, 19 (1977); ———, N. Munakata, S. Kitahara, *ibid.* **49**, 179 (1978).
4. Supported by grants in aid from the Ministry of Education, Science, and Culture and the Ministry of Health and Welfare, Japan. I thank M. Nagase for assistance.

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Sodium Channel Inactivation in Squid Axon is Removed by High Internal *p*H or Tyrosine-Specific Reagents

Abstract. *In squid axon, internal alkalinization from* pH *7.1 to* pH *10.2 results in a reversible decrease of the maximum inward current and the steady state sodium channel inactivation. Similar effects were observed after treatment of the axon with tetranitromethane or after iodination with lactoperoxidase. These results suggest that a tyrosine residue is an essential component of the inactivation process in this nerve.*

The molecular basis of excitability is poorly understood. Several lines of evidence suggest that sodium channels are sparsely distributed in the lipid membrane (*1*). Moreover, the sodium inactivation process appears to involve some proteinaceous component since internal perfusion of axons with proteolytic enzymes removes the inactivation (*2–4*). Armstrong and Bezanilla have proposed a model in which the inactivation process results from the plugging of open sodium channels by a mobile protein. This protein molecule is thought to be tethered to the inner surface of the membrane on one end and free to plug open sodium channels on the other end (*5*). Rojas and Rudy showed that after removal of the inactivation process with proteolytic enzymes, inactivation-like behavior could be reinstated by the addition of tetraethylammonium ion derivatives (*4*). They suggested that the plug of the Armstrong-Bezanilla model bears a positive charge on an amino acid residue of arginine or lysine. The last suggestion recently received additional support from the observation that glyoxal and diacetyl trimer, amino and guanidino group reagents blocked inactivation (*6*). In the experiments described here we tested the charged-tethered-plug model by varying the *p*H and titrating the specific amino acid groups involved in the inactivation process. Using the observed *pK* as a guide we then applied certain group-specific reagents to further confirm the presence of those amino acid residues.

Squid giant axons were provided by the Marine Biological Laboratory, Woods Hole, Massachusetts. Axons were dissected, cannulated, and perfused for 3 minutes with 1 mg of pronase grade B (Calbiochem) per milliliter of standard internal perfusate to remove the axoplasm. This procedure does not itself remove much of the inactivation (*2*). The axons were then internally perfused with 500 m*M* CsF, 5 m*M* NaF, and 20 m*M* buffer, or with 400 m*M* KF, 100 m*M* tetraethylammonium bromide (TEABr), 5 m*M* NaF, and 20 m*M* buffer. Both solutions blocked the voltage-dependent outward potassium currents. The voltage-dependent sodium currents could then be studied uncontaminated by potassium currents. The buffers used were Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pK_a 7.5 at 20°C; Bicine [*N,N*-bis(2-hydroxyethyl)-glycine], pK_a 8.3 at 20°C; CHES (cyclohexylaminoethanesulfonic acid), pK_a 9.5 at 25°C; and CAPS [3-(cyclohexylamino)-propanesulfonic acid], pK_a 10.4 at 20°C. In some experiments phenol red was added to the internal perfusate to ensure that the axon was adequately perfused and buffered. Experiments were run at temperatures between 0° to 10°C. The temperature in any given experiment was constant to within 0.2°C. Appropriate temperature corrections for the *p*H were made (*7*). The external perfusate was Woods Hole seawater at *p*H 8.2. Axons were voltage clamped by means of an axial wire piggyback electrode in an air-gap configuration (*8*). Series resistance compensation was employed.

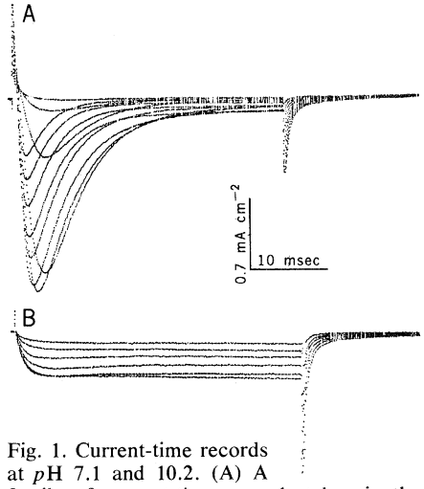

Fig. 1. Current-time records at *p*H 7.1 and 10.2. (A) A family of current-time records taken in the control solution containing 500 m*M* CsF, 5 m*M* NaF, and 20 m*M* Hepes buffer. The temperature-corrected *p*H was 7.1. The potential steps were −51, −40, −33, −23, −14, 10, 18, 28, 35. (B) The effect of increasing the *p*H to 10.2. The buffer was 20 m*M* CAPS. The number of voltage steps has been reduced for clarity (−40, −22, −13, −6, 2, 12). The holding potential was −60 mV with a conditioning pulse to −100 mV for 35 msec. The temperature was 3.7°C.

When the internal *p*H was raised from 7.1 to 10.2 a dramatic alteration of the sodium current was noted. Figure 1 illustrates two families of current-time records collected under voltage clamp conditions at normal and high *p*H. Two alterations by the *p*H 10.2 internal perfusate are noteworthy. First, the peak inward current was reduced by about 75 percent. Second, the sodium inactivation process was abolished, leaving the sodium current in an activated state at the end of the 45-msec pulse.

To further characterize the blockage of inactivation we applied a conditioning pulse of 70-msec duration and of varying magnitude immediately before the test pulse. The ratio of the peak inward current of the test pulse to the maximum peak inward current of the test pulse for large negative conditioning steps was plotted against the voltage of the conditioning pulse (Fig. 2). While the magnitude of the peak inward current of the test pulse was reversibly decreased by 50 percent at *p*H 9.9, the current during the test pulse was much less sensitive to the

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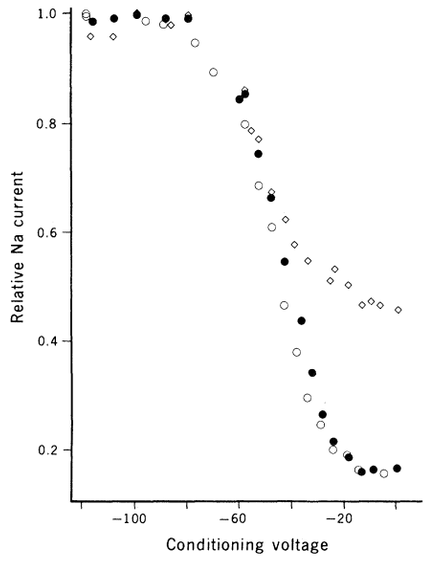

Fig. 2. The reversible removal of inactivation by high pH. A 70-msec conditioning pulse was given prior to a test pulse to 0 mV. The ratio of the test current for different conditioning potentials to the maximum test current at large hyperpolarizing conditioning pulses is plotted. Open circles show the control values obtained with an internal solution of 500 m*M* CsF, 20 m*M* Hepes, and 5 m*M* NaF at pH 7.1. The open diamonds show the effect of pH 9.9 obtained with a solution of 500 m*M* CsF, 20 m*M* CHES, and 5 m*M* NaF. The closed circles show the nearly complete recovery in the pH 7.1 control solution. The temperature was 4.0°C.

magnitude of the conditioning pulse than at pH 7.1. The removal of inactivation at pH 9 or 9.3 was much smaller. We estimate the apparent pK to be between 9.7 and 10.2; this range coincides with the titration range of the tyrosine phenolic group (*9*). The removal of inactivation by high pH was generally reversible, as shown in Fig. 2; however, reversibility was often hampered by an increase of the leakage conductance. The leakage conductance in the experiment of Fig. 2 doubled and returned to control values at pH 7.1. To our knowledge, high internal pH is the only reversible procedure for removing inactivation in squid giant axons. These results may partially explain the broadening of the action potential observed at internal basic pH (*10*).

Since a phenolic group of tyrosine was implicated by the apparent pK of the inactivation process, we perfused two reagents intracellularly to modify the phenolic group. The first, tetranitromethane (TNM), inserts a nitrate group on the three position of the phenolic ring. The effect of nitration is not only to insert a bulky group onto the aromatic ring, but also to shift the pK of the phenolic OH two to three pH units in the acid direction. Because the reaction rate is markedly increased at high pH, we applied the TNM in internal perfusate of pH 9 (*11*). The TNM was dissolved in ethanol and added to the internal perfusate. The final ethanol concentration was less than 1 percent and the TNM concentration was 50 μM. Records were taken before TNM application and after removal of TNM. Figure 3 illustrates pairs of current-time records at a given voltage before and after TNM treatment. After TNM treatment, the peak inward currents were reduced and the steady state current at the end of the depolarizing voltage step was increased. We interpret these results to indicate a decrease of maximum sodium conductance and a decrease of steady state inactivation. Thus, the effects of TNM resemble the effects of high pH and implicate a tyrosine residue in the inactivation process. While most selective for the phenolic ring of tyrosine residues, TNM also modifies to a lesser extent, sulfhydryl, carboxyl, thioether, indole, and certain vinyl groups. To further investigate the presence of a tyrosine residue, we used a procedure for the mono- and di-iodination of the phenolic group. A reaction mixture of 60 μg of lactoperoxidase (Calbiochem), 12 μg of glucose oxidase, 100 μg of glucose, and < 1 μg of NaI dissolved in 1.5 ml of internal perfusion solution at pH 7.3 was internally perfused for 15 minutes. The results were qualitatively similar to the effects of TNM. In addition to phenolic groups, imidazole groups may be iodinated as well (*12*).

Recently, Oxford *et al.* (*13*) examined the effects of a number of group-specific reagents on the sodium conductance of squid axon. Assuming that the reagents had access to their specific groups, they concluded that intact sulfhydryl, imidazole, amino, indole, and thioether groups are not necessary for proper function of sodium inactivation. In contrast, they found that *N*-acetylimidazole, which acetylates the phenolic oxygen of tyrosine, did block the inactivation process. They concluded on the basis of their experiments that a tyrosine group was involved in inactivation. In two experiments we have confirmed their observation, though we found the block to be incomplete. Moreover, α-chymotrypsin, which preferentially cleaves peptide bonds at the carboxyl side of tyrosine and phenylalanine residues, also abolishes inactivation (*3*). All these results further suggest that a tyrosine residue is involved in the inactivation process.

Tyrosine cannot be a candidate for the positively charged plug of the inactivation molecule, since at physiological pH the phenolic group is uncharged. To account for the time dependence of inactivation Rojas and Rudy (*4*) suggested that the inactivation process includes a hydrophobic group in addition to the positively charged blocking group. They proposed a phenylalanine residue in this role, but a tyrosine residue has a similar hydrophobicity (*14*). But, unlike phenylalanine, tyrosine can participate in hydrogen bonding (*15*). A model where inactivation is achieved through hydrogen bonding rather than hydrophobic interactions is suggested by the observation that acetylation of the phenolic group by *N*-acetylimidazole decreases inactivation, while possibly increasing the hydrophobicity of the aromatic ring. Other explanations are possible which involve steric factors or alterations of the charge structure of the sodium channel. Regardless of the model used to depict inactivation we conclude that an intact tyrosine residue is essential for the proper functioning of the inactivation process.

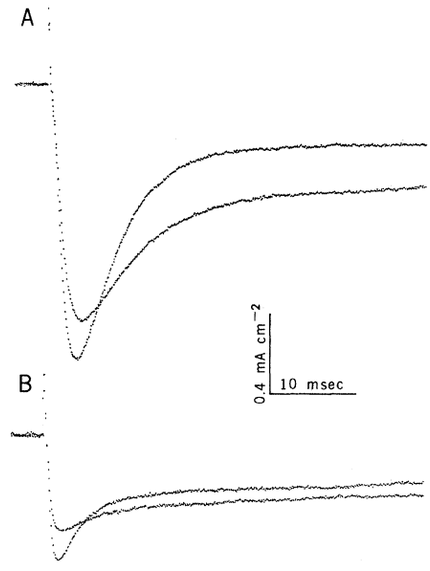

Fig. 3. The effect of tetranitromethane (TNM). (A and B) Pairs of currents at a given potential for a control record obtained in 400 m*M* KF, 100 m*M* TEABr, 20 m*M* Hepes, and 5 m*M* NaF at pH 7.1, and a record obtained after treatment in 400 m*M* KF, 100 m*M* TEABr, 20 m*M* CHES, and 5 m*M* NaF, with 50 μM TNM dissolved in ethanol (final concentration of ethanol was 1 percent) at pH 8.9. In (A) and (B) the control record has a larger peak inward current and a smaller steady state inward current than the TNM record; that is, the TNM record crosses the control record. The voltages of the steps in (A) and (B) are to −20 and 24 mV, respectively. The holding potential was −70 mV. The temperature was 7.5°C.

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References and Notes

1. C. M. Armstrong, *Q. Rev. Biophys.* **7**, 179 (1975).
2. _____, F. Bezanilla, E. Rojas, *J. Gen. Physiol.* **62**, 375 (1973).
3. C. Sevcik and T. Narahashi, *J. Membr. Biol.* **24**, 329 (1975).
4. E. Rojas and B. Rudy, *J. Physiol. (London)* **262**, 501 (1976).
5. C. M. Armstrong and F. Bezanilla, *J. Gen. Physiol.* **70**, 567 (1977).
6. D. C. Eaton, M. S. Brodwick, G. S. Oxford, B. Rudy, *Nature (London)* **271**, 473 (1978).
7. *Calbiochem Catalog* (Calbiochem, La Jolla, Calif., 1978), p. 163.
8. H. M. Fishman, *Biophysical Journal* **10**, 799 (1970).
9. C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.* **78**, 5287 (1956).
10. S. Terakawa and A. Watanabe, *Jpn. J. Physiol.* **26**, 693 (1976).
11. G. E. Means and R. E. Feeney, *Chemical Modification of Proteins* (Holden-Day, San Francisco, 1971).
12. D. R. Phillips and M. B. Morrison, *Biochemistry* **10**, 1766 (1971).
13. G. Oxford, C. Wu, T. Narahashi, *J. Gen. Physiol.* **71**, 227 (1978).
14. Y. Nazaki and C. Tanford, *J. Biol. Chem.* **246**, 2211 (1971).
15. J. P. Riehm *et al.*, *Biochemistry* **4**, 760 (1965).

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Cystic Fibrosis: Decreased Conjugation and Excretion of [^{14}C]Spermidine

Abstract. *Free and conjugated [^{14}C]spermidine were measured in plasma samples from normal individuals and cystic fibrosis patients. Within 4 minutes, the ^{14}C-labeled material in the plasma from normal individuals was 70 percent conjugated compared to no detectable conjugation by cystic fibrosis patients. Further, the patients excreted only 11 to 13 percent of the [^{14}C]spermidine in their urine within 72 hours whereas normal excretion was 60 to 76 percent. In both cases, the labeled material was in a conjugated form.*

Cystic fibrosis is an autosomal recessive genetic disorder characterized by pancreatic dysfunction and obstructive pulmonary disease (*1, 2*). The increased and abnormally viscous secretions of mucus that also characterize the disease may be a result of abnormalities in all exocrine glands (*2*). Although there have been several attempts to characterize this disease on a biochemical basis, the underlying metabolic defect remains obscure (*3*).

Several studies have shown that polyamine levels in whole blood are altered in cystic fibrosis patients (*4, 5*). Cohen *et al.* (*5*) found that concentrations of spermidine in erythrocytes were elevated to 150 percent as compared to those of controls, and that the spermine content in lymphocytes and granulocytes was decreased. Although Rosenblum *et al.* reported that all three polyamines (putrescine, spermidine, and spermine) were elevated 2- to 30-fold in the urines of patients with cystic fibrosis, patients who had been given a tracer dose of [^{14}C]spermidine excreted only 11 to 13 percent of the labeled material in their urines within 72 hours compared to 60 to 76 percent excreted by normal individuals in the same period (*6*). In this study we report that cystic fibrosis patients do not exhibit the rapid conjugation of [^{14}C]spermidine observed in normal individuals and patients with cancer (Fig. 1), conjugation that appears necessary for its excretion (*7*). These data support the proposition that polyamine metabolism is altered in, and related to, the pathology of cystic fibrosis.

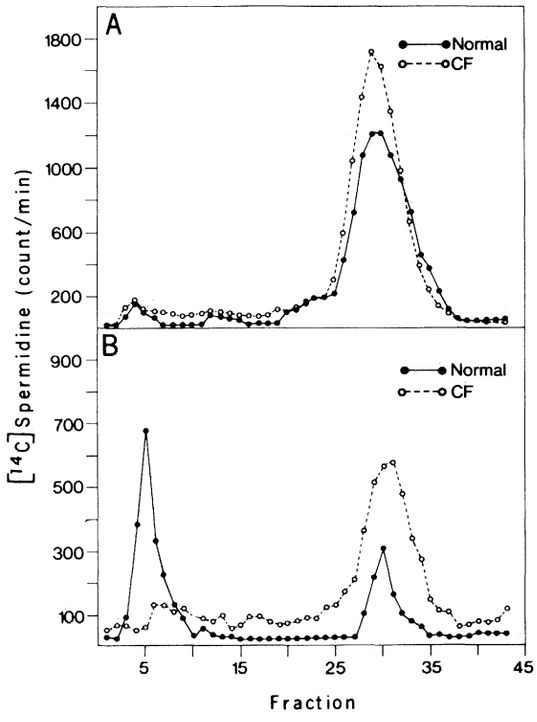

Fig 1. Dowex chromatography of plasma from normal and cystic fibrosis (*CF*) patients (A) 1 minute and (B) 4 minutes after intravenous [^{14}C]spermidine injection. The radiolabeled material was eluted with 2*M* to 5*M* linear NaCl gradient and collected in 1-ml fractions. Free [^{14}C]spermidine eluted in fractions 25 to 35, whereas conjugated [^{14}C]spermidine eluted in fractions 3 to 9.

Venous blood samples were taken at 1-minute intervals for 10 minutes after injection of [^{14}C]spermidine (100 μCi, 12.5 mCi/mmole). Total urine output was collected for 3 days after the injection. To separate free from conjugated [^{14}C]spermidine, samples of plasma or urine were chromatographed on Dowex 50W × 8 with a linear NaCl gradient (*8*). Chromatography of plasma samples from normal individuals (two male, two female, mean age 25 years) showed that, after 4 minutes, 70 percent of the [^{14}C]spermidine had been conjugated (Fig. 1). Cystic fibrosis patients (one male, one female, mean age 27 years, NIH clinical score <50) failed to demonstrate any conjugation after 4 minutes. Analysis of the labeled material in the urine showed that both normals and cystic fibrosis patients excreted more than 90 percent of the [^{14}C]spermidine in a conjugated form (data not shown). This suggests that conjugation, although delayed, does take place in cystic fibrosis patients. Sephadex G-25 chromatography of the polyamine conjugate isolated from normal individuals and cancer patients suggests that it has a molecular weight of 1000 (*9*).

On the basis of their studies of hepatectomized rats, Rosenblum and Russell (*8*) suggested that polyamine conjugation might occur in the liver. Although the occurrence of hepatic cirrhosis in cystic fibrosis has been well documented, the two patients we studied showed no clinical evidence of hepatic dysfunction. However, both cyctic fibrosis patients in this study received trimethoprim (Bactrim, Roche) therapy, and we cannot rule out the possibility that the delayed conjugation may be a drug effect, even though cancer patients receiving trimethoprim therapy did not have altered polyamine conjugation patterns.

It is not known whether delayed conjugation and sequestration of [^{14}C]spermidine represents a primary genetic expression of the disease or a secondary ef-

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