Studies on Basic Charged Molecules and Cell Membranes in Cystic Fibrosis[1] (35773)

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Recent studies on saliva (1) and sweat (2) from patients with cystic fibrosis (CF) have demonstrated a material or materials in CF secretions that inhibits sodium transport in the rat parotid gland (1, 2) and human sweat gland (3). An active inhibitory material is present in CF serum that disorganizes the rhythmic ciliary beat in rabbit trachea (4) and inhibits ciliary movement in the oyster (5) and fresh water mussel (6). The salivary material does not act by inhibition of membrane Na^+K^+ATPase (1), the most important enzyme involved in active sodium transport. Little else is known about these materials or their mechanism of action. One attractive hypothesis is that this material has a net positive charge. Positively charged molecules have a profound effect on cell surfaces, probably by effecting organizational changes in the lipid of the negatively charged membrane (7). Mangos and McSherry (8) have demonstrated that polycations, such as poly-L-lysine, inhibit sodium transport in the rat parotid gland in a manner similar to the active CF material. The sodium transport inhibition by CF saliva and poly-L-lysine may be neutralized by the negatively charged polyanion heparin (8). Bowman *et al.* (9) have suggested that the oyster inhibitory material in serum has a positive charge since it moves to the cathode with gamma globulin in starch gel preparations. This report describes our search for a unique polycation in CF saliva utilizing biological membrane assay systems affected by poly-L-lysine.

Materials and Methods. Patients with CF were taken from among those seen at the University of Wisconsin Cystic Fibrosis Clinic. All patients had a positive sweat test with signs and symptoms of the disease and all patients were receiving pancreatic replacement therapy. Patients and controls were given Parafilm to chew and mixed saliva was collected in a plastic container and centrifuged at 12,000*g* for 10 min.

^{14}C-succinate was purchased from the New England Nuclear Corp., Boston, Mass., ^{131}I-albumin from Squibb, New Brunswick, N. J., and ^{35}S-thiamine from Amersham Serle, Des Plaines, Illinois. Sarcoma 180 cells were a gift of Dr. R. Rueckert of the Department of Biophysics. All chemicals were reagent grade.

Acrylamide gel isoelectric focusing. 8% acrylamide was mixed with 200–300 μg saliva and ampholite, pH 3–10 (LKB), and the mixture was photopolymerized (10). The general procedure used in these studies was that of Dale (11). Gels were washed exhaustively in 10% trichloroacetic acid to remove the ampholite and stained with 0.05% Coomassie blue (12).

Inspection of the gels near the cathode end did not demonstrate an unusual basic protein with a high isoelectric point in CF saliva (Fig. 1). Five patients and six controls were tested. Gels obtained demonstrated 17–22 bands, depending on the protein concentration, and patterns of CF saliva were similar to controls.

Separation of mixed saliva and sweat proteins by gel electrophoresis at low pH. Acrylamide gel electrophoresis of CF and normal saliva has previously been reported with migration from the negative pole (cathode) to the positive pole (anode) at high pH (13, 14). In order to examine CF saliva for the

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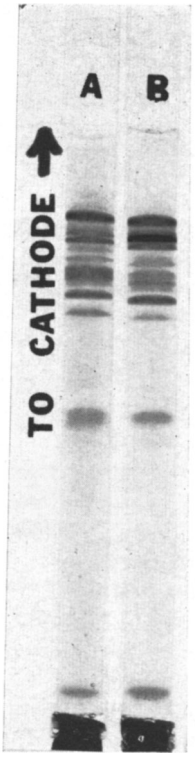

FIG. 1. Isoelectric pH focusing on acrylamide gels: ampholite range pH 3–10 with cathode (—) at the top; 300 μg of protein of normal saliva (A); and cystic fibrosis saliva (B) mixed with gel before photopolymerization; electrophoresis carried out for 15 hr at 110 V and 1–2 mA; gels stained with Coomassie blue.

presence of a unique positively charged peptide or protein it was necessary to carry out the migration to the cathode at low pH. 7.5% acrylamide gels, pH 4.5, were prepared by the method of Reisfeld *et al.* (15). Fifty μl of saliva, containing 100–250 μg of protein, were made hypertonic by the addition of solid sucrose and this solution was layered over the gel. A methylene blue marker was utilized. Migration was towards the cathode at 4 mA/gel. The gels were stained for protein with Coomassie blue (12).

Inspection of gels of salivary proteins from 10 patients and over 20 controls revealed 12–15 bands but did not demonstrate differences in CF and normal saliva (Fig. 2). A unique basic peptide or macromolecule was not demonstrated in CF saliva. Stained gels were scanned at a wavelength of 700 mμ with a Gilford gel scanner. Gel scanning was of value in demonstrating a basic peptide near the cathode end which was present in CF patients and controls and which frequently was not well demonstrated by visual inspection (Fig. 3).

1,4-^{14}C-Succinate oxidation by mitochondrial membranes. Rat liver mitochondria were prepared by differential centrifugation in 0.37 *M* sucrose, 0.05 m*M* EDTA, pH 7.4 (16, 17). Saliva from patients and controls were collected immediately before assay and dialyzed for 2 hr with two changes of 1000 ml of 0.001 *M* K_2HPO_4 buffer, pH 7.4. Decarboxylation of 1-4-^{14}C succinate was measured by collecting radioactive CO_2 produced in a center well containing paper saturated with Hyamine (16, 17). The reaction was stopped by the addition of 0.1 ml of 2 *M* H_2SO_4. The Hyamine-saturated paper con-

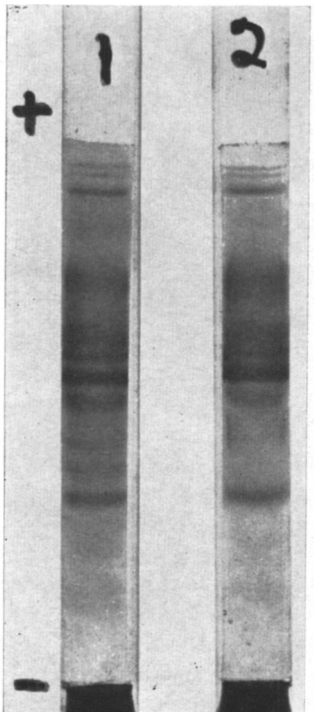

FIG. 2. Basic protein electrophoresis pH 4.5: 160 μg of normal saliva (1); and cystic fibrosis saliva (2) layered on top of gel in hypertonic sucrose; migration to bottom (cathode); gels stained with Coomassie blue.

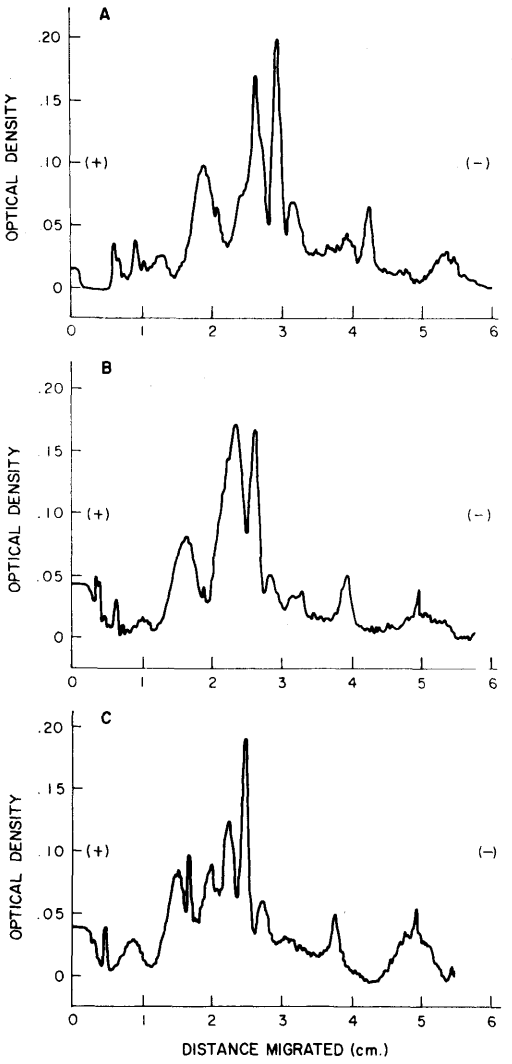

FIG. 3. Gel scan of stained acrylamide gels: 150 μg of protein of normal saliva (A); and CF saliva (B) layered over gels and electrophoresis carried out as described in Methods. A Gilford gel scanning device was used to scan Coomassie blue stained gels at 700 mμ. Scanning of gels revealed a basic peptide near cathode that was not readily visualized by inspection. (C) demonstrates increased resolution between 1–3 cm migration with normal saliva.

taining $^{14}CO_2$ was counted in a Packard liquid scintillation counter with Liquiflor–toluene.

Table I demonstrates that small amounts of polylysine stimulate succinate decarboxylation as previously described (16). Gamma globulin had a nonspecific inhibitory effect on succinate decarboxylation (Table I). Dialized CF saliva also inhibited this process. This inhibition was proportionally decreased on dilution. We interpret this to mean that the inhibition demonstrated by CF saliva is nonspecific and related to the increased protein concentration in CF saliva (26, 27).

^{131}I-Albumin uptake by sarcoma 180 cells. The general procedures of Ryser were used to determine ^{131}I-albumin uptake by sarcoma 180 cells (18, 19). Dialyzed ^{131}I-albumin, cystic fibrosis saliva (0.3 ml/3 ml of culture media), or polylysine at 10^{-7} *M* were incubated with about 5×10^6 cells for varying lengths of time. Attached cells were washed with Hanks' media, trypsinized, centrifuged, washed in Hanks' media, centrifuged, and the uptake of ^{131}I-albumin into the cells determined (17).

Polylysine at 10^{-7} *M* concentration had a marked enhancing effect on the uptake of ^{131}I-albumin by sarcoma 180 cells in culture (Fig. 4). Cystic fibrosis saliva, 0.3 ml/3 ml of culture media, did not have this effect.

Thiamine-^{35}S release by cultured human fibroblasts. Recently it has been demonstrated that drugs which affect active movement of sodium across membranes in the central nervous system may also stimulate the efflux of ^{35}S-thiamine from cells (20, 21). We have tested polylysine, normal saliva and CF saliva on this effect in cultured human cells. About 5×10^4 attached fibroblasts were incubated with 2.5–5.0 μCi of ^{35}S-thiamine in F_{12} media with 15% fetal calf serum for 24–26 hr. Cells were washed five times with Hanks' solution, incubated 1 hr in Hanks' solution and washed three times before incubation with saliva or poly-L-lysine. Released thiamine was counted by liquid scintillation. Poly-L-lysine had a marked effect in the release of ^{35}S; CF saliva did not have this effect (Table II).

Discussion. The fundamental defect responsible for cystic fibrosis (CF) has not been demonstrated. Several recent observations, such as the metachromasia of CF fibroblasts (22), and the increased mucopolysaccharide (23) and glycogen content (24) of CF fibroblasts are secondary findings related

TABLE I. Effect of Cystic Fibrosis and Normal Saliva, Polylysine and Gamma Globulin on 1-4-^{14}C-Succinate Oxidation in Mitochondria.

Values given are ± 1 SD. Saliva was dialized in 0.001 *M* K_2HPO_4, pH 7.8, at 4° for 2 hr and added in a volume of 0.3 ml/1 ml assay.

Test substance	Inhibition (%)	Stimulation (%)
Normal saliva (15)	21.8 ± 18.7	
Cystic fibrosis saliva (4)[a]	69.5 ± 7.5	
(2)[b]	30.1	
Polylysine, 10 μg		80
Gamma globulin, 0.5 mg	42	

[a] $p < .01$ compared to normal saliva.

[b] Diluted by one-third to give protein concentrations in same range as normal controls; $p > .05$ compared to normal saliva.

to an unknown primary abnormality. Other findings in the pathology of CF may also be secondary. Increased bradykinin content of CF saliva (25) is as likely related to the general increased content of organic material in CF saliva (26, 27) as it is to the pathogenesis of the disease. Decrease in β-glucuronidase activity in sweat glands of patients with CF (28) is not found in cultured fibroblasts from affected patients (29). Pancreatic replacement therapy may complicate the pathophysiology of CF. Pancreatic enzymes increase the size of secretory glands and cause abnormalities in viscosity, protein, and ion content of secretions in experimental animals (30). Beta adrenergic agents such as isoproterenol duplicated some of the pathology of CF in rats (31), and this plays an unknown role in the clinical and chemical findings. It is not possible at present to determine the importance of several recent observations in erythrocytes of CF patients. These include decreased sodium efflux (32), decreased Ca^{2+}ATPase (33), increased glucose-6-phosphate dehydrogenase activity and decreased NADPH/NADP ratios (34) in red cells from affected patients.

This study began as an attempt to find an assay for the sodium inhibitory material in CF saliva since we felt identification of this

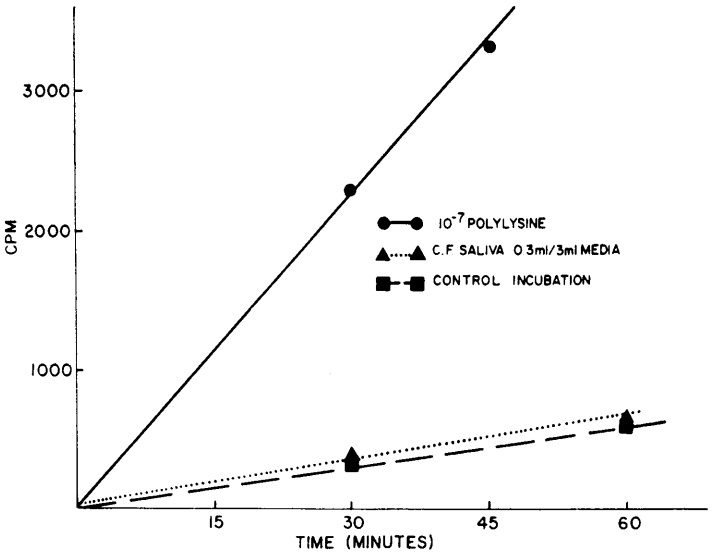

FIG. 4. Uptake of ^{131}I-albumin by Sarcoma 180 cells in culture: procedure as described in Methods; polylysine, 10^{-7} *M* or CF saliva 0.3 ml/3 ml of media were added to incubation media.

TABLE II. Release of ^{35}S-Thiamine by Cultured Human Cells.

Fibroblasts in log phase were grown in F_{12} with 15% fetal calf serum and 2.5–5.0 μCi of ^{35}S-thiamine for 24–26 hr. Polylysine, normal saliva and CF saliva was incubated with the washed cells for 10 (A) and 20 (B, C) min. Data are expressed in counts per minute of ^{35}S released above a control incubation ± 1 SD with number of observations in parentheses.

Expt.:	A	B	C
CF saliva			
Mean	218 ± 17 (2)	313 ± 351 (4)	300 ± 118 (3)
Control saliva			
Mean	248 ± 12 (2)[a]	330 ± 202 (5)[a]	481 ± 217 (4)[a]
Polylysine			
2 μg	1170	1200	

[a] $p > .05$ compared to control saliva.

material would enable us to identify primary and secondary factors in this disease. The assay systems available, that of retrograde injection into rat parotid glands (1, 2) and human sweat glands (3) are difficult to master and not amenable to the large number of analyses required if isolation were attempted. Several assay systems were then tested for possible use based on the hypothesis that this material has a positive charge. Poly-L-lysine, a basic polypeptide, is also a strong inhibitor of sodium transport in the rat parotid gland (8), accelerates the uptake of ^{131}I-albumin by sarcoma 180 cells (19), stimulates the oxidation of succinate by isolated rat liver mitochondria (15), and effects the release of thiamine from cultured human fibroblasts (Table II). We find that saliva from patients with CF does not act like poly-L-lysine under similar conditions. The acrylamide gels and acrylamide isoelectric focusing also did not reveal a positively charged polypeptide or protein unique to CF saliva (Fig. 1, 2). All patients were receiving pancreatic replacement therapy, which alters the patterns of basic secretory proteins in experimental animals (30). Since CF salivary protein patterns were similar to controls, this suggests CF saliva does not contain a unique basic protein or polypeptide in amounts greater than 3 μg/100 μl of saliva, and it also suggests that pancreatic replacement has no effect on basic secretory proteins in these patients.

Studies with biological assay systems, such as rabbit (4), oyster and mussel cilia (5, 6), and sodium transport in the rat parotid (1, 2, 8) and the membrane assay systems reported here, suffer the disadvantage that they are not specific for the active CF material. Ouabain, as well as poly-L-lysine, affects rabbit cilia (4) and sodium transport in the rat (1, 8). From this it might be expected that selection of basic proteins by ion exchange chromatography would affect the assay systems available. We find that basic proteins isolated by ion exchange chromatography from normal saliva stimulates succinate oxidation in isolated rat liver mitochondria (29). Although the systems described here did not detect the presence of a unique basic polypeptide or protein in unfractionated CF saliva, such a search should not be discontinued. The active serum dyskinesis factor may have a positive charge (9). Identification of such a material in CF saliva would shed light on the metabolic defect in CF and might suggest a rational approach to therapy.

Summary. A recent hypothesis is that unknown positively charged molecules are involved in the pathophysiology of cystic fibrosis. ^{131}I-albumin uptake by sarcoma 180 cells in culture, 1-4-^{14}C-succinate oxidation by isolated mitochondria and ^{35}S-thiamine released by cultured fibroblasts are affected by poly-L-lysine, a positively charged polypeptide. CF saliva does not have this effect. Polyacrylamide gel electrophoresis and acrylamide isoelectric focusing suggests that basic secretory proteins are normal in CF saliva. Conventional pancreatic replacement therapy does not affect basic charged secretory proteins as it does in experimental animals.

1. Mangos, J. A., McSherry, N. R., and Benke, P. J., Pediat. Res. 1, 436 (1967).

2. Mangos, J. A., and McSherry, N. R., Science **158**, 135 (1967).

3. Kaiser, D., Drack, E., and Rossi, E., Lancet **1**, 1003 (1970).

4. Spock, A., Heick, H. M. C., Cress, H., and Logan, W. S., Pediat. Res. **1**, 173 (1967).

5. Bowman, B. H., Lockhart, L. H., and McCombs, M. L., Science **164**, 325 (1969).

6. Besley, E. T. N., Patrick, A. D., and Norman, J. P., J. Med. Genet. **6**, 278 (1969).

7. Butler, K. W., Smith, I. C. P., and Schneider, H., Biochem. Biophys. Res. Comm. **40**, 770 (1970).

8. Mangos, J. A., and McSherry, N. R., Pediat. Res. **2**, 378 (1968).

9. Bowman, B. H., McCombs, M. L., and Lockhart, L. L., Science **167**, 871 (1970).

10. Fawcett, J. S., Fed. Eur. Biochem. Soc. Lett. 1, 81 (1968).

11. Dale, G., and Latner, A. L., Lancet **1**, 847, (1968).

12. Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J., Anal. Biochem. **20**, 150 (1967).

13. Mandel, I. D., Kutscher, A., Denning, C., Thompson, R., and Zegarelli, Arch. Dis. Child. **113**, 431 (1967).

14. Gugler, E., Pallavicini, J. C., Swerdlow, H., and Di Sant'Agnese, P. A., J. Pediat. **71**, 585 (1967).

15. Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Nature **195**, 281 (1962).

16. Rasmussen, H., Shirasu, H., Ogata, E., and Hawker, C., J. Biol. Chem. **242**, 4669 (1967).

17. Fain, J. N., Scow, R. O., and Chernick, S., J. Biol. Chem. **238**, 54 (1963).

18. Ryser, H. S. P., Lab. Invest. **12**, 1009 (1963).

19. Ryser, H. S. P., and Hancock, R., Science **150**, 501 (1965).

20. Itokawa, Y., and Cooper, J., Biochem. Pharmacol. **18**, 545 (1969).

21. Itokawa, Y., and Cooper, J. R., Science **166**, 759 (1969).

22. Danes, B. S., and Bearn, A. G., Lancet **1**, 1061 (1968).

23. Matalon, R., and Dorfman, A., Biochem. Biophys. Res. Commun. **33**, 954 (1968).

24. Pallavicini, J. C., Wiesmann, U., Uhlendorf, W. B., and Di Sant'Agnese, P. A., J. Pediat. **77**, 280 (1970).

25. Lieberman, J., and Littenberg, G., Pediat. Res. **3**, 571 (1969).

26. Chernick, W. S., Barbero, G. J., and Parkins, F. H., J. Pediat. **59**, 890 (1961).

27. Di Sant'Agnese, P. A., Grossman, H., Barling, R. C., and Denning, C. R., Pediatrics **22**, 567 (1958).

28. Gibbs, G. E., and Griffin, E. D., Science **167**, 993 (1970).

29. Benke, P. J., unpublished results.

30. Mangos, J. A., Benke, P. J., and McSherry, N., Pediat. Res. **3**, 562 (1969).

31. Mangos, J., McSherry, N., Benke, P., and Spock, A., *in* (D. Lawson, ed.), "5th International Cystic Fibrosis Conference," p. 25. C. Nicholls, London (1969).

32. Balfe, J. W., Cole, C., and Welt, L. C., Science **162**, 689 (1968).

33. Horton, C. R., Cole, W. Q., and Bader, H., Biochem. Biophys. Res. Comm. **40**, 505 (1970).

34. Shapiro, B. L., Lee, S. M., and Warwick, W. J., Biochem. Biophys. Res. Comm. **39**, 816 (1970).

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