Cystic Fibrosis: Isolation and Physical Properties of a Salivary Cystic Fibrosis Factor

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Summary

A ciliostatic factor has been isolated from cystic fibrosis (CF) saliva by dialyzing it from purified α -amylase prepared by a glycogen-complex method. This method of isolating the CF factor is an improvement over the previously employed heparin procedure. The activity of the isolated factor is proportional with concentration using the oyster gill ciliostatic assay and in its inhibition of mammalian glycogen debranching enzyme. The ciliostatic action of the factor can be reversed by heparin under certain conditions. The type of inhibition of the debranching enzyme by the isolated CF factor indicates that its chemical structure is similar to that observed with hydroxyalkylamines and polyamine metabolites. Physical properties of the isolated factor indicate that it is of low molecular weight and is labile as a function of pH and temperature. At neutral pH the conditions under which it is maintained have a direct effect on the length of time that it is stable.

Speculation

These studies indicate how the stability of the ciliostatic CF factor isolated from saliva is related to the physical conditions to which it is exposed. This information should be of value in evaluating its activity as well as in investigations into its chemical nature.

Cystic fibrosis is a genetic disease inherited as an autosomal recessive trait (14, 25, 32). The primary metabolic defect(s) remains unknown. In 1967 Spock *et al.* (40) described a rabbit tracheal ciliary dyskinesia factor found in the serum of CF patients. Since then, additional factors have been reported as present in the biologic fluids and/or cultured fibroblasts from CF patients and their obligate heterozygotes. These factors have in general been described as immunoglobulins, complement components, abnormal proteins, peptides ranging from 1,000-10,000 in molecular weight, as well as abnormal enzymes and polyamines and/or their metabolites (7, 8, 10-12, 15, 20, 22, 29, 31, 33, 35). Various bioassays have been developed for the detection of these CF factors and include the oyster gill ciliary assay developed by Bowman *et al.* (7) and modified by Schmoyer *et al.* (39), a fresh water mussel assay by Besley *et al.* (4), a modification of the original rabbit tracheal assay (40) described by Conover *et al.* (9), the retrograde perfusion assay by Mangos (29-31), and its modification as described by Taylor *et al.* (41), and recently a short-circuit current membrane system by Araki *et al.* (1) adapted from Asano (2). In addition, a means of detection utilizing isoelectric focusing has been described by Wilson and Fudenberg (43).

Although a ciliostatic factor from the saliva of CF patients has been shown to be associated with α -amylase (15, 16), attempts to detect differences between CF and normal amylase in terms of their catalytic activity have been unsuccessful (5, 35). However, the ciliostatic activity can be separated from purified CF α -amylase by dialysis, indicating that the factor is a

low molecular weight molecule ($\leq 12,000$) bound to the amylase (20, 35).

The current method for obtaining this salivary CF factor from α -amylase utilizes the heparin precipitation method of Doggett and Harrison (15, 16) as extended by Gillard *et al.* (20). The factor, although ciliostatic, was detected by an enzymatic assay which utilized its ability to inhibit muscle glycogen debranching enzyme (20). As opposed to the oyster gill bioassay, this enzymatic method is quantitative and not subject to the variability of the ciliostatic method (14, 43). The heparin method employed by Gillard *et al.* (20) for obtaining the isolated factor is, however, highly variable in terms of yield.

The purpose of this study was to develop a more reproducible method of isolating the CF factor from salivary α -amylase so as to investigate its physical properties.

MATERIALS AND METHODS

COLLECTION OF SALIVA

Saliva was donated by CF patients at the Cystic Fibrosis Center at the Texas Institute for Rehabilitation and Research, with the control saliva samples being obtained from research associates and their children who had no family history of CF. Samples (ca. 10-20 ml) of whole mixed saliva were collected without stimulatory aids to promote secretion, and large collections were obtained through pooling of a moderate population of subjects. Upon collection, the saliva was treated as described.

ANALYTIC PROCEDURES

α -Amylase (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) activity was measured by the dinitrosalicylate method as described previously (20). Absorbance was measured at 540 nm using a Gilford 240 spectrophotometer. Amylase activity is expressed in terms of milligrams of maltose liberated in 3 min at 30° (3). Total carbohydrate was determined using the phenol sulfuric acid method (18). Protein was determined using an adaptation (37) of the Miller modification (34) of the Folin-Lowry method (27) with a bovine serum albumin as a standard. Mammalian debranching enzyme amylo-1,6-glucosidase/4- α -glucanotransferase (dextrin 6- α -glucosidase, EC 3.2.1.33/1,4- α -D-glucan; 1,4- α -D-glucan 4- α -glycosyltransferase, EC 2.4.1.25) was measured using glycogen phosphorylase limit dextrin (LD) as a substrate in the static assay described by Nelson *et al.* (37).

The oyster gill ciliostatic bioassay for the CF factor was used as described by Doggett and Harrison (15, 16) employing the procedure of Bowman *et al.* (7) as modified by Schmoyer *et al.* (39). Recently the oysters have been unpredictable on a daily basis in their response to the effect of the CF factor, aside from their usual seasonal (summer) unresponsiveness. Batches of about six oysters were obtained daily throughout the spring months from a local distributor and tested for ciliary response. Out of every four or five batches, usually two or three oysters from one particular batch were found to give the typical response

reported previously (6, 15, 16, 20). Ciliostatic time was measured as the time in minutes taken to inhibit ciliary activity. Careful selection of oysters was made as described above on the basis of ciliary and nonciliary inhibition using as controls normal human serum, CF serum, oyster juice, 0.5 M NaCl (sea water salinity), phosphate buffer (pH 6.9) in 0.5 M NaCl, and various molar concentrations of diaminoethylethylpropane (DAHP) in 0.5 M NaCl (pH 6.9). DAHP, a stable synthetic hydroxyalkylamine, is an inhibitor of the debrancher and also ciliostatic (20). It was used as a standard of known ciliostatic time. All oyster gill incubations in normal serum, oyster juice, 0.5 M NaCl, 0.01 M DAHP solutions below 0.01 M in 0.5 M NaCl (pH 6.9), and 0.1 M phosphate buffer in 0.5 M NaCl (pH 6.9) were nonciliostatic and used routinely as negative controls. All oyster gill incubations in CF serum, solutions above 0.125 M DAHP in 0.5 M NaCl (pH 6.9), and above 0.15 M phosphate buffer in 0.5 M NaCl (pH 6.9) were ciliostatic and used routinely as positive controls. All solutions with a ciliostatic time greater than 60 min were considered nonciliostatic (12).

pH determinations were made with an IL model 245 pH meter equipped with an automatic temperature compensation probe. pH values cited for solutions were determined at 25°, not the temperature of usage unless noted otherwise. All buffers and other salts were in the sodium or chloride form unless specified. Dialysis tubing was presoaked to remove preservatives and other materials before use.

ISOLATION OF CF FACTOR

The CF factor was isolated from whole saliva collected as described. The isolation procedure was the same for individual or pooled samples. The overall procedure for a typical pooled batch of ca. 100 ml was as follows.

Whole saliva was centrifuged at $5000 \times g$ for 15 min at 4°. All subsequent steps were carried out at 4° unless noted. Absolute ethanol was added to a concentration of 40% v/v, with constant stirring, dropwise at a maximum rate of 0.5 ml/min. The precipitate formed was centrifuged for 20 min at $9000 \times g$ and discarded. To the supernatant, 0.2 M phosphate buffer (pH 8.0) containing 0.01 M NaCl was added to a final concentration of 0.01 M. To this is added 0.1 ml of a 2% aqueous glycogen (shellfish) solution/ml starting whole saliva, followed immediately by additional ethanol to a final concentration of 40% v/v. Stirring was maintained throughout, and continued for 30 min after the last addition; the mixture was then centrifuged at $6000 \times g$ for 15 min. The precipitate of the enzyme-glycogen complex was washed twice with cold 40% ethanol containing 0.01 M phosphate buffer + 0.01 M NaCl (pH 8.0). Approximately 2 ml buffered ethanol was added/mg glycogen for the first wash and 1.0 ml/mg glycogen for the second wash.

The washed precipitate was suspended at room temperature in 0.02 M phosphate buffer (pH 6.9) containing 0.01 M NaCl and incubated at 30° for 1 hr to digest glycogen. Additional aliquots of buffer were added at 15-min intervals during digestion until all remaining precipitate was dissolved. The pH was then slowly adjusted at room temperature to 8.5 with 1 N ammonium hydroxide and the mixture centrifuged at 4° for 10 min at $8000 \times g$. The small precipitate formed was discarded and the pH of the supernatant was readjusted to 7.0 by addition of 1 M acetic acid. The mixture was kept in an ice bath at 0° for ca. 60 min at which time a white flocculant precipitate formed. The solution was centrifuged at $13,000 \times g$ for 20 min and washed once with 40% ethanol containing 0.01 M phosphate buffer + 0.01 M NaCl (pH 8.0) and recentrifuged. The supernatant was discarded. The washed precipitate is then resuspended in 0.02 M phosphate buffer (pH 6.9) containing 0.5 M NaCl.

The dissolved suspension is then transferred to a dialysis sack and dialyzed using a rocking dialyzer. The suspended material was dialyzed against 2.5–3.0 vol distilled water, changed three

times at equal intervals. Dialysis was continued for 6 days. The final dialysate solution was lyophilized and stored at -20° .

The lyophilized dialysate was dissolved in distilled water and its effect on debranching enzyme was determined as described previously (20) by the addition of 10 to 100 μ l of the dialysate solution to a total of 200 μ l in the standard limit dextrin static assay described by Nelson *et al.* (37). Controls containing 10–100 μ l H₂O and/or buffer were used in place of the dialysate solution. The ciliostatic activity of samples was also measured as described previously (20).

MATERIALS

Purified rabbit muscle glycogen debranching enzyme (amylase-1,6-glucosidase/4- α -glucanotransferase) was prepared according to the method of Watts and Nelson (42). Glycogen phosphorylase limit dextrin was prepared as described previously (37). Additional materials for the debrancher assay were obtained as described previously (42). Soluble potato starch used for routine α -amylase assays was obtained from Merck and Co., Rahway, NJ. Corn starch used for precipitation studies of amylase was obtained from B&A Specialty Chemicals Division, Allied Chemical Corp., Morristown, NJ. Defatted corn starch was prepared by *n*-butanol extraction of this material. Maltose and oyster (shellfish) glycogen were obtained from Nutritional Biochemicals, Cleveland, OH. 3,5-Dinitrosalicylic acid was obtained from Eastman Kodak, Rochester, NY. DAHP and hydroxyethylpropanolamine (HEPA) were obtained as described elsewhere (21). All other biochemicals and chemicals were of the highest commercial grade available. Standard grade dialysis tubing was obtained from Arthur H. Thomas, Philadelphia, PA.

RESULTS

The first isolation of a ciliostatic CF factor from salivary α -amylase was described by Gillard *et al.* (20) using an extension of the method of Doggett and Harrison (15, 16) for producing a heparin-amylase precipitate from saliva. The isolated factor was found to inhibit the action of muscle glycogen debranching enzyme, and this was used to develop an enzymatic assay for the factor (20). The difficulty with the heparin isolation method was that it gave small and inconsistent yields of factor. This appeared to be related to the purity of the amylase obtained.

In order to improve the yield and purity of the amylase other methods of amylase complex precipitation were tried. Amylase is well known to form complexes with dextrans that are precipitable in the presence of ethanol and this has been used to remove amylase as a contaminant in the purification of other enzymes. Schramm and coworkers have developed a method for producing highly purified amylase based on its formation of a complex with glycogen in the presence of ethanol (24, 28). A study was conducted using normal saliva to determine whether glycogen precipitation of amylase in the presence of 40% ethanol was more efficient than using soluble potato starch or defatted corn starch. As can be seen from Figure 1, glycogen is a better adsorbent than either soluble potato starch or defatted corn starch. Not only is less glycogen required for maximum adsorption but precipitation of the complex is nearly quantitative at 2 mg/ml. The amylase purification procedure based on the formation of a glycogen-amylase complex reported by Loyter and Schramm (28) was then employed with slight modifications using glycogen at 2 mg/ml. The final procedure is described in the experimental section.

Comparative isolations of the CF factor from amylase were done using both the heparin method described by Gillard *et al.* (20) and the glycogen complex method for purifying amylase adapted from Loyter and Schramm (28). Samples of whole saliva from individual CF patients, a sample of pooled saliva from different CF patients, and samples from normal individuals were used. Each sample was processed by both the heparin and the glycogen methods. The recovery and specific activity of the α -amylase obtained by both methods was determined. In addi-

tion, the degree of inhibition of the debranching enzyme by the CF factor isolated from the amylase obtained from both methods was determined. A representative example of the results from both CF patients and normal individuals is shown in Table 1. As can be seen, the glycogen precipitation method produced uniformly higher yields. The specific activities are comparable with those reported in the literature for salivary α -amylase purified to homogeneity as are the yields (19, 28). In contrast the specific activities of the heparin method are widely variable and indicate on the average only ca. 30% purity. The yields of amylase are also uniformly very low. It is obvious that the glycogen precipitation method is superior in both purity and yield of amylase to the heparin method. The CF factor isolated by dialysis of the glycogen method purified amylase produced a greater quantity of inhibitory material as measured by the debrancher assay when compared on an equivalent dilution basis (Table 1). As seen (Table 1) all concentrated heparin as well as glycogen dialysates containing the factor inhibited the debranching enzyme. However, as noted in the table, the heparin dialysates were 10 times more concentrated in volume and 4 times as much was used in the standard inhibition assay.

Fig. 1. Glycogen, soluble potato starch and defatted corn starch precipitation of α -amylase from normal whole saliva in the presence of 40% ethanol. Precipitate curves indicate the amount of amylase precipitated by each dextrin in the presence of 40% ethanol. Supernatant curves indicate the amount of amylase remaining in solution after dextrin precipitation. The standard amylase assay was employed as described in text.

Thus, on an equivalent basis in terms of the quantity of factor in solution, 40 times more heparin method-isolated factor was used than glycogen method-isolated material to produce the inhibition shown. This would indicate that from the same amount of whole saliva, the glycogen method produced ca. 40 times more isolated CF factor than the heparin method. Because of the lack of direct chemical quantitation of the factor at present, yields are determined solely by its inhibitory effect on the debranching enzyme. Since the same starting volumes were treated identically the glycogen method is obviously also superior in terms of consistency and yield of isolated factor.

When it was apparent that randomly pooled CF saliva gave the same yield of isolated factor as did individually treated samples (Table 1), pooled saliva samples were used to prepare the isolated factor. CF patients were selected and whole saliva samples were obtained and pooled for a total volume of ca. 100 ml. The method of purification and isolation of the CF factor by dialysis from the pooled saliva was performed in the same manner as described for the individual patient samples.

Although the CF factor isolated by the glycogen method was more consistent and produced larger yields of inhibitory material as measured by the debrancher inhibition assay, the question arose as to whether this isolated material was ciliostatic as had been demonstrated previously for the material isolated by the heparin method (20). In order to test this, the glycogen isolated CF factor was subjected to the oyster gill ciliostatic assay. The oyster bioassay was employed as described in the experimental section. Although the infrequency of response of the bioassay made it unreliable as a routine method for analysis, oysters were found which gave the typical response reported previously (7, 15, 16, 20). When oysters were found that were responsive they were used to test the ciliostatic activity of the factor isolated by the glycogen method.

As shown in Figure 2, increasing ciliostatic time was observed to be a linear function of dilution of the factor to the point of no longer being ciliostatic. It is of interest to note that a greater than 2-fold extension in ciliostatic time was also observed by increasing the dilution of CF factor with a 1.0% solution of heparin (Figure 2). Varying heparin concentrations with and without the factor was done to determine the optimum reversals of ciliostatic inhibition as well as to see what if any effect heparin alone had on the ciliostasis (Fig. 3). A 1:20 dilution of the CF factor in heparin at concentrations below 1.0% indicated a strong reversal in ciliostatic time, whereas heparin alone

Table 1. Comparison of heparin and glycogen isolation of salivary CF factor

Source	Type	Whole saliva				α -Amylase fraction						Isolated CF factor debrancher inhibition, % ¹
		Vol, ml	Total protein, mg	Total units	Sp act, U/mg	Method	Vol, ml	Total protein, mg	Total units	Sp act, U/mg	Recovery, %	
<i>JLE</i> (15 yr, F) ⁴	CF	4.5	13.37	7,047	527	Heparin	1.0	0.26	232	892	3.3	56 ²
						Glycogen	1.5	2.85	6,176	2,167	88.0	64 ³
<i>JRC</i> (11 yr, M)	CF	4.0	10.50	6,282	598	Heparin	1.0	0.31	177	571	2.8	81
						Glycogen	1.5	2.39	5,884	2,461	94.0	64
<i>JSC</i> (14 yr, M)	CF	4.0	8.08	4,832	598	Heparin	1.0	0.19	8.0	42	0.2	74
						Glycogen	1.5	1.59	3,328	2,093	69.0	76
<i>CM</i> (20 yr, F)	CF	4.0	8.04	5,430	675	Heparin	1.0	0.50	300	600	5.5	72
						Glycogen	1.5	2.28	4,366	1,914	80.4	69
<i>PS</i> (19 yr, F)	CF	4.0	10.44	6,763	648	Heparin	1.0	0.32	409	1,278	6.0	33
						Glycogen	1.5	2.33	5,380	2,309	80.0	74
Pooled (8–15 yr, M, F)	CF	4.0	8.32	5,530	665	Heparin	1.0	0.39	298	764	5.4	61
						Glycogen	1.5	2.10	4,285	2,040	77.5	55
<i>JI</i> (26 yr, M)	Normal	4.5	7.47	5,027	673	Heparin	1.0	0.11	63	573	1.3	None
						Glycogen	5.0	3.25	4,161	1,280	83.0	None

¹ Inhibition values are percentage in standard assay.

² All heparin dialysates were concentrated to 0.50 ml, 100 μ l used in standard assay.

³ All glycogen dialysates were concentrated to 5.0 ml, 25 μ l used in standard assay.

⁴ Parentheses: age in years and sex.

Fig. 2. Oyster ciliary inhibition by the isolated CF factor. Each curve represents identical aliquots of factor: one diluted in 0.5 M NaCl, and the other in a solution of 1.0% heparin in 0.5 M NaCl. Ciliostatic time was measured as described in the text as the time in minutes necessary to stop oyster cilia activity.

became ciliostatic above a concentration of *ca.* 1.0% (Fig. 3). Optimum reversal of ciliostatic time occurred in the presence of factor at 0.5% heparin.

The effect of the concentration of the CF factor on the inhibition of mammalian debranching enzyme was determined, as shown in Figure 4, by addition of 1–75 μ l of the isolated CF factor to a total volume of 200 μ l in the standard limit dextrin static assay (37). The results indicate a linear relationship up to *ca.* 60–70% inhibition. A double reciprocal plot of inhibition of the debrancher by the isolated CF factor indicates it to be a reversible noncompetitive inhibitor (Fig. 5). This type of inhibition is the same as that previously observed with hydroxyalkylamine inhibitors of the glucosidase site on the debrancher using glycogen phosphorylase limit dextrin as a substrate (21, 36, 38). This type of kinetic behavior suggests that the isolated CF factor is a molecule having similar structural characteristics.

Further substantiation of the structural similarity is indicated in Table 2. As can be seen, all of the polyamines and their hydroxy analogs had an inhibitory effect on the oyster gill ciliostatic assay, whereas only the hydroxy analogs HEPA and DAPH inhibited the debrancher. This is probably due to the fact that the analogs resemble the structure of the various hydroxyalkylamines, such as Tris (trihydroxyaminomethane) that have been shown to inhibit the debrancher, whereas the polyamines, spermine, spermidine, and cadaverine, do not (21, 36–38). It is well known that many polycationic materials such as poly-L-lysine will cause ciliostasis (7). The results in Table 2 thus indicate that the inhibition of the debranching enzyme is more specific in terms of chemical structure.

The physical properties of the isolated CF factor were investigated to determine its stability with time, temperature, and pH. The temperature stability of the CF factor with preincubation as a function of time is shown in Figure 6. The results indicate that the inhibitory activity of the CF factor is affected only slightly at 20° and 30° for 2 hr or if incubated longer than 2 hr at 40°, whereas the activity incubated for 15 min at 45° is significantly affected. Incubation at 50° and 55°, respectively, showed *ca.* 25–30% loss after 15 min; total loss of activity occurred between 18–20 min and after *ca.* 2 min at 60°.

Storage of the isolated CF factor at reduced temperatures as a function of time was also investigated in order to retain optimum activity. The stability of the factor dissolved in 0.02 M phosphate buffer (pH 6.9) plus 0.007 M NaCl stored at room temperature, 4°, and frozen is shown in Fig. 7. No difference was observed using distilled water rather than buffer. In addition no difference was observed in the frozen material, stored as a solution, dried by rotary evaporation at 30° bath temperature or as the lyophilized powder. Further studies were performed

using varying concentrations of NaCl. Optimum stability occurred at a NaCl concentration of 0.5 M NaCl and remained constant at each of the storage temperatures (room temperature, 4°, –20°) when measured after 14 days. Enzymatic stability of the factor was measured daily as before over a 14-day period (Fig. 7). Under these conditions, with daily freezing and thawing for assay, there was a loss of less than 5% of the total activity. The isolated factor was also found to be soluble in anhydrous methanol. In comparison to the low ionic strength aqueous systems (Fig. 7), the factor stored in methanol retained a greater percentage (*ca.* 50%) of its activity with time.

Five-minute incubations of the isolated factor were done at various pH values at room temperature, using normal purified salivary α -amylase as an enzymatically active protein for comparison (Fig. 8a). The results indicate a somewhat broader pH stability than amylase with an optimum for the factor around neutral pH. A further study was performed at various pH values with time using the isolated factor (Fig. 8b). Maximum factor stability again occurred around neutral pH, with inactivity rapidly increasing with time at *ca.* 2 pH units or more on either side of neutrality. It is of interest to note that the presence of 0.5 M NaCl had no effect on stability as a function of pH (Fig. 8b).

Fig. 3. Reversal of oyster ciliary inhibition by the isolated factor with varying concentrations of heparin in 0.5 M NaCl. Normal human serum, oyster juice, and 0.5 M NaCl were used as negative controls. Three diagnosed CF patients and the isolated factor diluted 1:20 with 0.5 M NaCl without heparin were used as positive controls.

Fig. 4. Effect of concentration of the isolated factor on the inhibition of debranching enzyme. The standard limit dextrin assay was used as described in text. The incubation mixture contained 1–75 μ l isolated factor to a total volume of 200 μ l in standard limit dextrin static assay described in the text. Percentage of inhibition was measured as the decrease in the amount of glucose produced by the enzyme in the presence of the factor.

Fig. 5. Double reciprocal plot of inhibition of the debrancher by the isolated CF factor. The standard procedure was used as described in the text. Incubation mixtures were prepared for each inhibitor concentration and contained the same quantity of enzyme. Aliquots were removed at 2-min intervals. The velocities shown represent extrapolated initial velocities.

Table 2. Effect of polyamines on debrancher and oyster gill assays

Inhibitor	Structure	Cilio-static time, ¹ min	% Debrancher inhibition (LD) ²
Cadaverine	$H_2N(CH_2)_5NH_2$	8	None
Spermidine	$H_2N(CH_2)_3NH(CH_2)_4NH_2$	7	None
Spermine	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	4	None
HEPA	$HO(CH_2)_3NH(CH_2)_2OH$	24	30
DAPH	$H_2N(CH_2)_3NH(CH_2)_2OH$	14	19 ³
0.5 M NaCl		60	

¹ All compounds were 0.05 M in 0.5 M NaCl, adjusted to pH = 7.0.

² All inhibitors were 0.05 M in the reaction mixture, adjusted to pH 6.8, standard limit dextrin (LD) assay.

³ Value from Gillard *et al.* (20).

Identical aliquots of isolated factor in 0.02 M sodium phosphate, pH 6.9 + 0.007 M NaCl containing a predetermined inhibitory activity on the debranching enzyme were incubated for 10 min at room temperature in new, precleaned, disposable Corning Pyrex glass test tubes (18 × 150 mm) and new 15-ml sterile polystyrene test tubes. Each tube was gently vortexed three times throughout the 10 min period. The aliquot was then transferred to a successive test tube and the process repeated nine times (180 min). There was no detectable loss in factor inhibitory activity in the aliquots exposed to either the glass or polystyrene test tubes over the time interval indicated. In addition, the final transfer aliquot was allowed to remain in both the glass and plastic tubes for 24 hr at 4° and was again assayed for inhibitory activity. After 24 hr the loss in activity was *ca.* 10% for both glass and plastic. This is the same loss in 24 hr as in Figure 7 where repeated transfers were not performed. These results indicate that there is little or no detectable loss of factor activity due specifically to contact with either glass or plastic surfaces.

DISCUSSION

The procedure developed for isolating a CF factor from whole saliva utilizes the fact that the factor is bound to α -

amylase and remains so through subsequent purification of the enzyme. The larger yield of the isolated factor obtained using the glycogen complex method as opposed to the heparin complex method is more than likely related to the larger recovery

Fig. 6. Temperature stability of the CF factor with preincubation as a function of time. The standard limit dextrin assay was used as described in text. Percentage of inhibition was measured as the decrease in the amount of glucose produced by the enzyme in the presence of factor. The factor was preincubated in 0.02 M sodium phosphate, pH 6.9, containing 0.007 M NaCl.

Fig. 7. Stability of the isolated factor at reduced temperatures under various conditions as a function of time. Lyophilized aliquots of comparable inhibitory activity were employed and taken up in the systems shown. —: aliquots assayed each day; ---: aliquots stored for 14 days before assay. The material at -20° was stored frozen as the liquid. The material at -90 was lyophilized and stored as a dry powder for 14 days before assay.

Fig. 8. Stability of the isolated factor as function of pH and time. *a*: aliquots of the isolated factor were preincubated for 5 min at the various pH values shown at room temperature, neutralized, and then assayed for activity using the standard debrancher assay described in the text. Aliquots of normal purified salivary α -amylase were treated in a similar manner and were assayed as described in the text. *b*: aliquots of the isolated factor were preincubated at room temperature for the times shown, neutralized, and then assayed for activity using the standard debrancher assay as described in the text. $\times$: values obtained under identical conditions in the presence of 0.5 M sodium chloride.

and higher purity of the amylase produced. The overall recovery of the isolated factor cannot be determined by the enzymatic method used to assay it since the presence of α -amylase in the saliva interferes with the substrate used in the debranching enzyme assay. The total amount of factor present in whole saliva and whether it is all bound to amylase, are therefore not known. The present results also do not indicate whether there is more than one ciliostatic factor in CF saliva.

The fact that CF factor isolated by the glycogen method is a potent ciliostatic agent indicates that it is similar to the factor previously isolated by the heparin method (20). The isolated material is also comparable in chemical composition to the material isolated by the heparin method as previously described (20). The ciliostatic activity of the isolated factor as measured by the oyster gill bioassay also indicates that it is related to the genealogy of CF and is not found in other chronic lung disease states (17, 26).

The increase in ciliostatic time produced by the isolated factor in the presence of heparin is of interest. Since the isolated factor has basic or cationic groups (20), it may be electrostatically bound to the heparin which is anionic. This effect has been observed for heparin and other polyanions when CF factors from sweat and saliva have been tested using the Mangos retrograde perfusion method (29, 31) and is explicable on the basis of their postulated cationic character (13, 30). The heparin effect is also consistent with the *in vivo* and *in vitro* findings of Doggett and Harrison (15, 16) who used *ca.* 1% heparin with CF serum with the oyster gill ciliostatic assay. It is apparent, however, that the effect of heparin on the oyster gill assay is complex and not simply an effect on the factor since concentrations of heparin alone exceeding 1% were found to be increasingly ciliostatic. The addition of excess calcium ion did not negate the effect of heparin alone on the cilia and the addition of EDTA rather than heparin did not reduce the ciliostatic activity of the factor. Whether *in vivo* effects of *ca.* 1% heparin observed on increasing the ciliostatic time of CF serum (16) are due to interaction with the factor alone cannot be ascertained from the present results.

The type of inhibition produced by the isolated factor on the debranching enzyme is of importance since it gives an additional indication of the chemical nature of the factor. The noncompetitive inhibition observed using limit dextrin as a substrate is the same as that observed with a series of hydroxyalkylamines such as Tris, which act as reversible substrate model inhibitors of the debrancher's glucosidase activity (21, 36–38). These hydroxyalkylamines apparently "mimic" an activated structure of glucose formed by the enzyme act as "transition state" analogs by binding to the debrancher's glucosidase site (21, 36). Such analogs are the only known reversible inhibitors of the debrancher aside from glucose and debranched limit dextrin which are the products of its action on limit dextrin. The noncompetitive inhibition shown by the isolated factor suggests that it has a hydroxyalkylamine-like structure. This is consistent with the suggestion that the isolated CF factor is some type of polyamine metabolite (20, 35). As can be seen from the comparison of the effects of various polyamines and their deaminated hydroxyalkyl analogs on the inhibition of the debrancher and oyster gill ciliostasis, the debrancher is more specific in terms of which structures cause inhibition. The polyamines spermine, spermidine and cadaverine, do not affect the debrancher although they do cause ciliostasis. It is known that a large number of amines such as polylysine (7) will cause ciliostasis, therefore the effect of all the polyamines and analogs on ciliostasis is not surprising nor is it as indicative of the chemical nature of the factor as is the more specific inhibition of the debrancher.

The physical properties of the isolated CF factor indicate that it is labile when exposed to extremes of pH and temperature, although by comparison on a temporary basis, no more so than α -amylase. Unlike α -amylase, however, the isolated factor is not stable to storage for extended periods of time even under refrigerated conditions except at -90° . The effect of relatively high concentrations of NaCl (0.5 M) in prolonging stability cannot be explained at the present time. The lability of the isolated factor indicates that it is chemically a fairly reactive molecule. If the material is a polyamine metabolite such as an aminoaldehyde as has been suggested (20, 35), this type of reactivity would be expected. It is known that compounds of this type tend to form a variety of chemical condensation products under relatively mild conditions (23).

The lability properties of the isolated CF factor and its

instability under various storage conditions may explain some of the discrepancies in measurement and reproducibility of activity reported in the past for various CF factors (6, 14, 44). Certainly the physical properties reported here should be taken into account in the future.

CONCLUSION

A ciliostatic CF factor has been obtained from saliva using an improved method of isolating it in a relatively pure form from α -amylase. The type of inhibition of mammalian debranching enzyme observed suggests its chemical nature. The physical properties of the factor indicate that it is a low molecular weight compound which is quite labile to variations in pH and temperature. Its stability under mild conditions depends on the manner in which it is maintained.

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