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Calcium
cystic fibrosis
inhibitors
inorganic phosphate
parotid saliva
protein
turbidity

Colloid and Crystal Formation in Parotid Saliva of Cystic Fibrosis Patients and Non-Cystic Fibrosis Subjects. I. Physicochemistry

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Extract

Two types of turbidity were found in parotid saliva from both cystic fibrosis (CF) patients and non-CF subjects. On cooling saliva, a rapidly forming, reversible, cold-dependent turbidity appeared in increasing amounts with decreasing temperature and increasing protein concentration. At 37°, a slowly forming, stable turbidity appeared in increased amounts in parotid saliva samples containing increased amounts of calcium. The 2° centrifuged pellet consisted predominantly of protein, whereas the 37° pellet contained calcium, inorganic phosphate, and protein. The cold-dependent turbidity at 2° was not inhibited by EDTA, but 37° turbidity was dramatically inhibited. Urea and guanidine hydrochloride reduced 2° turbidity, and, to a lesser extent, inhibited 37° turbidity. The tendency towards higher levels of protein, amylase, and calcium in CF compared with child control parotid saliva (4, 6) causes a greater incidence and degree of turbidity formation in saliva of CF patients. In this paper only the nature of the turbidity has been investigated, not its relative occurrence in each group of subjects.

Speculation

In cystic fibrosis, a tendency towards high levels of calcium and protein in parotid saliva would mediate towards deposition of colloidal protein and calcium phosphate within parotid gland ducts and on tooth surfaces adjacent to duct orifices. A similar oversecretion of calcium and protein in other CF exocrine secretions may result in obstruction by stagnant or cooled secretions.

Parotid saliva from patients with CF and control subjects is clear as collected, but saliva with elevated protein (amylase) and calcium concentrations becomes turbid on standing (4). Cystic fibrosis patients tend to have parotid saliva with higher amylase and calcium concentrations than age-matched child control subjects (4, 6), and turbidity formation is therefore encountered more frequently and to a greater degree in parotid saliva of CF patients. Aspects of physicochemical properties of this turbidity formation, particularly in relation to temperature and to protein, calcium, and phosphate concentrations, are recorded in this paper, and electron microscope appearance and electrophoretic properties of the insoluble material are reported in a subsequent paper (2). In this paper only the nature of the turbidity has been investigated, not its relative occurrence in each group of subjects.

SUBJECTS AND METHODS

SALIVA COLLECTION

Subjects for the study were 13 cystic fibrosis patients aged 8–13 years (attending the Cystic Fibrosis Clinic at the Royal Alexandra Hospital for Children), and 7 young adult non-CF subjects aged 18–24 years (hospital staff). To some extent subjects were selected from those known to have a high parotid saliva flow rate and high protein and calcium concentrations, in whom the saliva had been noted in previous studies (4, 6, 7) to become turbid on standing at room temperature.

Saliva was collected using a modified Lashley cup attached by suction over the orifice of Stenson's duct. Stimulation was 2 drops of 5% citric acid on the tongue every 30 sec for periods up to 10 min, which gave sufficient volume for tests (at least 3 ml). Informed consent was obtained from adult subjects and from parents of children in this study.

TURBIDITY MEASUREMENT

The turbidity of parotid saliva was measured as the optical density of a sample in a quartz semi-microcuvette (path length 1 cm) at 320 nm in a Gilford 240 spectrophotometer. Cuvettes incubated at temperatures below room temperature were coated with Calotherm liquid demister (nonultraviolet absorbing) before reading to prevent water condensation on the outside of the cold cuvette.

Cold-dependent changes in the turbidity of the saliva were measured after 10 min of temperature equilibration of aliquots of saliva in cuvettes in water or ice water baths at temperatures of 37°, 25°, 20°, 15°, 5°, and 2°. Results were expressed as the optical density difference at 320 nm between the reading at temperature (T) and the initial optical density at 37° ($\Delta OD_{320}^{T,37}$). To give a thermodynamic relationship, the optical density difference was plotted against the inverse of temperature ($1/T \times 10^4$ °K $^{-1}$).

Reversibility of cold-dependent turbidity was assessed by changing saliva samples at 10-min intervals from 37° to 2° and back to 37° several times, and comparing OD_{320} with the readings of aliquots maintained at 37° and 2°.

The turbidity at 37° was determined by measuring OD_{320} of aliquots of parotid saliva incubated in cuvettes at 37° for various time periods up to 2 hr, and results were expressed as the optical density difference between the reading at time (t) and the initial optical density at zero time (ΔOD_{320}^{t-0}).

ADDITION OF INHIBITORS OF TURBIDITY

Paired aliquots of parotid saliva (0.9 ml) were made up to 1.0 ml with (*1*) water as control; (*2*) solutions of EDTA (pH 7.0) to give final concentrations of 0.5, 1, 2, or 3 mM EDTA; or (3) solid urea or guanidine hydrochloride to give final concentrations of 1 or 6 M.

The optical density at 320 nm (OD_{320}) was read at 37°, then one of the pair of duplicates was incubated at 37° and the other at 2° for 2 hr, and ΔOD_{320}^{t-0} and $\Delta OD_{320}^{T,37}$ were measured.

CHEMICAL ASSAYS

For chemical assays of turbid material, aliquots of saliva were centrifuged at the incubation temperature at 4,500 rpm for 10 min. The supernatant was removed and the centrifuged pellet was dissolved in 4 M hydrochloric acid to half the original volume. Both pellets and uncentrifuged parotid saliva (for total concentrations) were assayed for protein, calcium, and inorganic phosphate content.

Protein was assayed by the method of Lowry *et al.* (21), with tartrate being replaced by citrate (15), and pellet samples were neutralized with 4 M sodium hydroxide before assay. Calcium was determined by atomic absorption spectrophotometry (16) with samples diluted 1:10 in lanthanum chloride (La 5,000 ppm). Inorganic phosphate was measured using acid-ammonium molybdate-ascorbic acid reagent (12). Pellet results were expressed as concentration in the original saliva volume.

RESULTS

Two types of turbidity were found in parotid saliva from both CF patients and non-CF subjects, with no apparent qualitative differences between CF and non-CF saliva. On cooling saliva, a rapidly forming, reversible, cold-dependent turbidity appeared in increasing amounts with decreasing temperature and increasing protein concentration. At 37°, a slowly forming, stable turbidity appeared in increased amounts in parotid saliva samples containing increased amounts of calcium. Aspects of physicochemical behavior of turbidity formation on cooling below 37° ($\Delta OD_{320}^{T,37}$) and with time at 37° ($\Delta OD_{320}^{T,0}$) were investigated.

COLD-DEPENDENT TURBIDITY

The cold-dependent turbidity formed immediately on cooling parotid saliva to 2°, and disappeared instantly on rewarming to 37° (Fig. 1), demonstrating the reversible nature of the complex causing turbidity.

The turbidity increased rapidly with decreasing temperature, forming two types of temperature-dependence curves (Fig. 2): one being exponential (*A*) and the other having a central plateau region (*B*). The graph of $\log \Delta OD_{320}^{T,37}$ *vs* $1/T(°K^{-1}) \times 10^4$ (Fig. 3) was linear for *A* and consisted of two intersecting linear regions ("bent") in *B*.

There was a direct relationship between the temperature-dependent turbidity change and the number of particles causing light scattering and hence also for the equilibrium constant (K) of colloid formation. Using the Van't Hoff thermodynamic relationship $\left(\frac{d\log K}{d\,1/T} = \frac{-\Delta H}{2.303R}\right)$ the positive linear slope of the plot ($\Delta OD_{320}^{T,37}$ *vs* $1/T(°K^{-1})$) was proportional to the enthalpy change (ΔH) for turbidity formation, i.e., colloid formation involved loss of energy ($\Delta H < 0$). Since the system was at equilibrium ($\Delta G = 0$, $\Delta G = \Delta H - T\Delta S$), then the entropy change (ΔS) as colloid formed was negative, indicating production of a more ordered system. The linear "bent" lines may have been due to particles coalescing rather than the formation of new aggregated particles.

The amount of turbidity formed at lower temperatures (10°, 5°, and 2°) was proportionally related to total protein concentration in the parotid saliva (Table 1). There was no relationship with total calcium or inorganic phosphate.

37° TURBIDITY

In contrast to the cold-dependent turbidity, at 37° a slower process of turbidity formation occurred with time and continued to rise for 2 hours or more (Fig. 4). At lower temperatures, an increase in turbidity with time was slower, although cooling also caused an immediate increase in OD_{320} because of the formation of cold-dependent turbidity. At 37° the amount of turbidity produced at different time intervals increased with increasing parotid saliva total calcium concentrations (Table 2). There was no significant correlation between turbidity and total inorganic phosphate or protein concentrations.

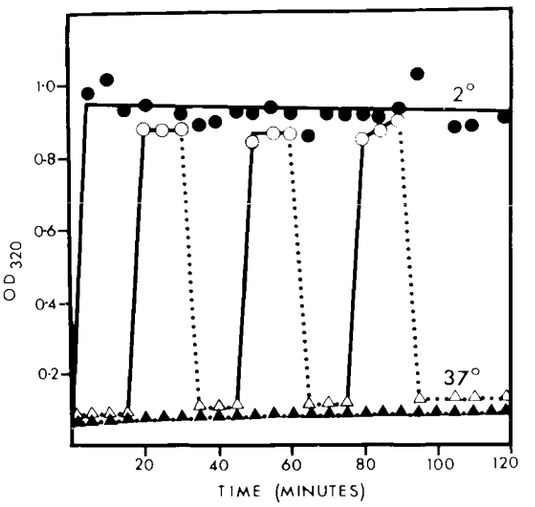

Fig. 1. Reversibility of cold-dependent turbidity formation in parotid saliva of a cystic fibrosis patient (calcium 0.87 mM; protein 365 mg/100 ml). Turbidity (OD_{320}) is plotted against time. Saliva kept at 37° (▲); saliva kept at 2° (●). Saliva changed from 37° (△) to 2° (○) several times showing formation and clearing of turbidity.

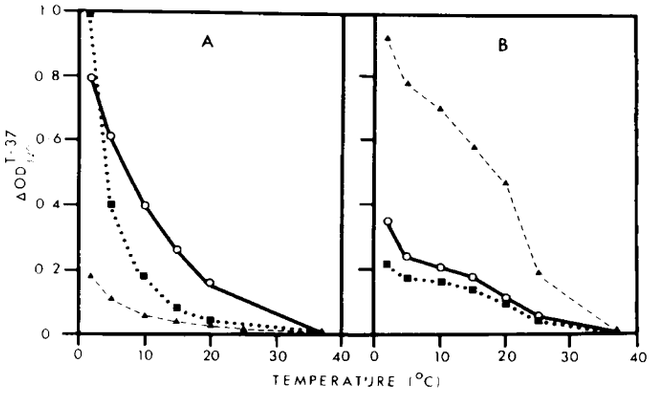

Fig. 2. Temperature dependence of cold turbidity in parotid saliva. Turbidity, expressed as the change in OD_{320} at temperature (T) compared with reading at 37° (ΔOD_{320}^{T-37}), is plotted against temperature (T). *A*, exponential curves; *B*, curves with central plateau.

Figure	Symbols	Cystic fibrosis (CF) or Non-CF	Calcium, mM	Protein, mg/100 ml
2*A*	○——○	CF	0.75	230
	■····■	Non-CF	1.03	255
	▲ ▲	Non-CF	0.85	165
2*B*	▲ ▲	Non-CF	0.96	225
	○——○	CF	1.17	250
	■····■	CF	0.66	150

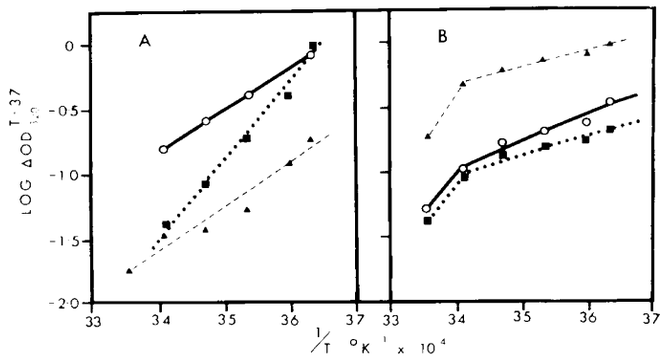

Fig. 3. The Van't Hoff relationship for cold-dependent turbidity formation plotted as log ΔOD_{320}^{T-37} against inverse of temperature ($1/T\ °K^{-1} \times 10^4$). *A*, from exponential curves of Figure 2*A*; *B*, from curves with central plateau of Figure 2*B*.

Table 1. *Dependence of cold turbidity on protein concentration*

X	Y	Temperature, °C	No.	*r*	*P*
Total protein	ΔOD_{320}^{T-37}	25	18	0.26	NS[1]
		20	20	0.19	NS
		15	20	0.34	NS
		10	19	0.46	<0.05
		5	20	0.50	<0.05
		2	19	0.65	<0.01

[1] Not significant.

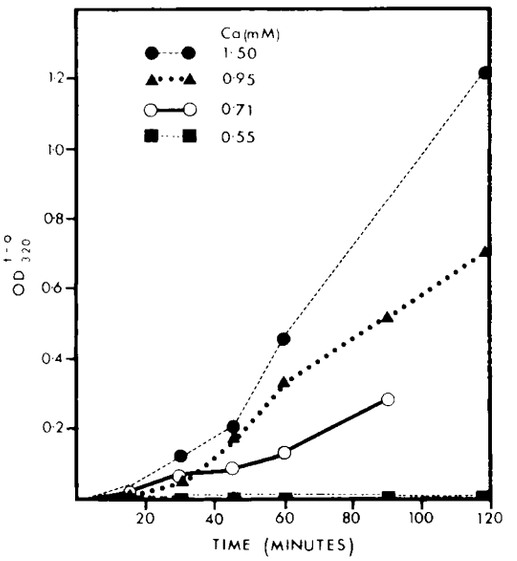

Fig. 4. Turbidity formation in parotid saliva at 37°. Turbidity, expressed as the change in OD_{320} at time (t) compared with reading at zero time (ΔOD_{320}^{t-0}), is plotted against time (t).

Symbols	Cystic fibrosis (CF) or non-CF	Calcium, mM	Protein, mg/100 ml
● ●	CF	1.50	490
▲····▲	CF	0.95	450
○——○	CF	0.71	200
■ ■	Non-CF	0.55	80

Table 2. *Dependence of 37° turbidity on calcium concentration*

X	Y	Time at 37°, min	No.	*r*	*P*
Total calcium	ΔOD_{320}^{T-37}	15	20	0.37	NS[1]
		30	22	0.61	<0.001
		45	20	0.58	<0.01
		60	22	0.72	<0.001
		90	10	0.65	<0.05
		120	6	0.77	NS

[1] Not significant.

COMPOSITION OF PELLETS FROM PAROTID SALIVA AFTER INCUBATION AT 2° AND 37°

After incubation at 2°, the centrifuged pellet consisted predominantly of protein, with only small quantities of calcium and inorganic phosphate (Table 3). In contrast, in the 37° pellet there were statistically significantly ($P < 0.001$) increased levels of calcium and inorganic phosphate, with approximately the same level of protein (Table 3). The Ca/P ratio of the 37° pellet was also significantly ($P < 0.001$) greater than that of the 2° pellet.

EFFECT OF TOTAL CALCIUM CONCENTRATION ON PELLET COMPOSITION

The total calcium concentration of the parotid saliva was significantly related to pellet calcium and protein concentrations in the 2° and 37° pellets, and pellet phosphate concentration in the

Table 3. *Composition of pellets from parotid saliva after incubation at 2° for 10 min and at 37° for 2 hr*[1]

Temperature, °C	No.	Protein, mg/100 ml	Calcium, mM	Inorganic phosphate, mM	Ca/P ratio
2°	23	7.8 ± 5.2	0.04 ± 0.03	0.08 ± 0.03	0.49 ± 0.36
37°	22	10.8 ± 9.6	0.22 ± 0.18	0.20 ± 0.14	0.97 ± 0.34
P		NS	<0.001	<0.001	<0.001

[1] Pellet concentrations were calculated per volume of saliva, and expressed as mean ± SD. NS: not significant.

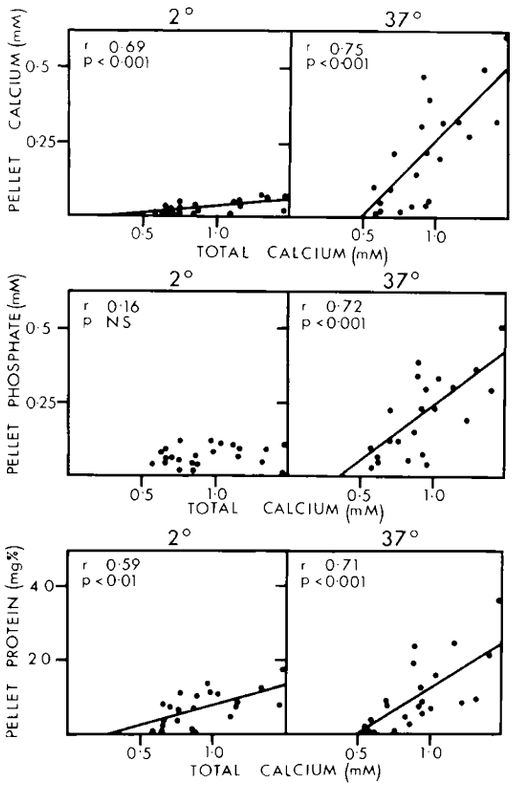

Fig. 5. The relationship between parotid saliva total calcium concentration and centrifuged pellet calcium, inorganic phosphate, and protein content after 10 min at 2° and after 2 hr at 37°. Cystic fibrosis and non-cystic fibrosis subjects.

37° pellet (Fig. 5). The regression lines at 2° were appreciably lower than those at 37° (Fig. 5).

EFFECT OF TOTAL PROTEIN AND PHOSPHATE CONCENTRATIONS ON PELLET COMPOSITION

Parotid saliva total protein correlated positively with pellet protein concentration at 2° ($P < 0.001$) and 37° ($P < 0.01$) (Table 4). Total protein did not correlate with pellet calcium or phosphate.

Parotid saliva total inorganic phosphate concentration bore no significant relationship to pellet components at either temperature.

INTERRELATIONSHIPS OF PELLET COMPONENTS

At 2°, inorganic phosphate and protein deposition in the pellet were positively related ($P < 0.05$), and neither of these components was related to calcium in the pellet (Fig. 6). After incubation at 37°, however, calcium in the pellet was positively correlated with both pellet protein ($P < 0.001$) and inorganic phosphate ($P < 0.001$), as was pellet protein with pellet inorganic phosphate ($P < 0.001$) (Fig. 6).

Table 4. *Relationships between total protein concentration and pellet composition at 2° and 37°*

		2°			37°		
X	Y	No.	*r*	*P*	No.	*r*	*P*
Total protein	Pellet calcium	23	0.37	NS[1]	22	0.33	NS
	Pellet phosphate	23	0.07	NS	22	0.34	NS
	Pellet protein	23	0.78	<0.001	22	0.65	<0.01

[1] Not significant.

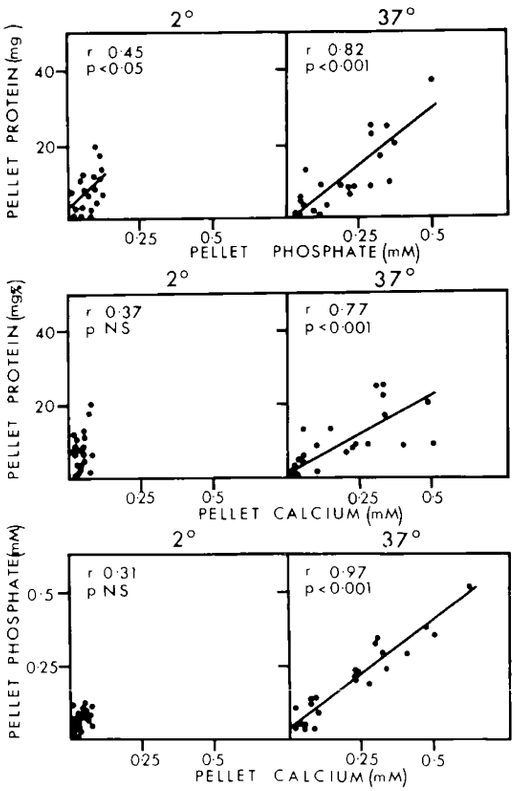

Fig. 6. Interrelationships between parotid saliva centrifuged pellet contents of calcium, inorganic phosphate, and protein after 10 min at 2° and after 2 hr at 37°. Cystic fibrosis and non-cystic fibrosis subjects.

EFFECT OF EDTA ON PELLET COMPOSITION

The production of cold-dependent turbidity at 2° was not inhibited by the addition of EDTA (0.5 3 mM). The protein concentration deposited at 2° remained approximately the same, whereas calcium and inorganic phosphate in the pellet remained low or decreased.

In contrast, 37° turbidity was dramatically inhibited by the presence of EDTA in the same concentration range. With EDTA addition the difference in OD_{320} after 2 hr remained at zero, in contrast to rising levels in the controls. The 37° pellet calcium and inorganic phosphate levels were lowered to levels close to zero, and pellet protein concentration in most cases was lowered significantly in the presence of EDTA.

EFFECT OF UREA AND GUANIDINE HYDROCHLORIDE ON PELLET COMPOSITION

Both urea (1 and 6 M) and guanidine hydrochloride (1 and 6 M) reduced cold-dependent turbidity at 2° by lowering the pellet protein, calcium, and inorganic phosphate concentrations. The effect was greater for guanidine hydrochloride than for urea.

Urea and guanidine hydrochloride at 6 M concentrations also inhibited 37° turbidity, whereas there was less inhibition at 1 M concentrations. Inhibition was evident as decreased change in OD_{320} and diminished pellet content after 2 hr incubation at 37° when compared with controls.

DISCUSSION

These studies of citric acid-stimulated parotid saliva have disclosed formation of two different types of turbidity, one forming preferentially at 2° and the other at 37°. The 2° turbidity was rapidly forming, was reversible on rewarming, contained colloidal protein, and correlated positively with parotid saliva protein concentration. The 37° turbidity formed slowly, was stable, contained a calcium-phosphate-protein complex, and correlated positively with parotid saliva calcium concentration.

Although both types of turbidity may occur in saliva of both CF patients and non-CF subjects, the tendency towards higher levels of protein, amylase, and calcium in CF in comparison with child control parotid saliva (4, 6) causes a greater incidence and degree of turbidity formation in saliva of CF patients.

COLLOID FORMATION IN PAROTID SALIVA

The reversible nature of the cold-dependent turbidity formation suggested a physical equilibrium of aggregating saliva components demonstrating a temperature-sensitive bonding. This was further confirmed by the temperature-dependence of the equilibrium constant for the complex. The increase in cold turbidity formation with increasing saliva protein concentration and its independence of total calcium and phosphate concentrations indicated that the process might involve aggregation of parotid protein. The composition of the 2° pellets supported this theory since they contained appreciable amounts of protein and only small amounts of calcium and phosphate. The addition of EDTA in amounts sufficient to chelate all divalent ions had no effect on reducing either turbidity or pellet protein concentration. Electron microscopy of turbid material at 2° showed amorphous, round particles ranging in size from 0.1 to 20 μm (2, 4). Thus the cold turbidity appeared to be colloidal aggregates of parotid proteins formed in increased amounts in saliva with elevated protein concentrations, and independently of the availability of calcium.

The Van't Hoff relationship for turbidity formation indicated that energy was lost ($\Delta H < 0$) during protein aggregation, while the negative entropy change ($\Delta S < 0$) suggested that the process produced a more ordered arrangement of proteins. There was no correlation between slopes of lines obtained in the plot of log ΔOD_{320}^{T-47} *vs* $1/T(°K^{-1})$ and total calcium, protein, and phosphate concentration or the group of subjects used, i.e., the energy requirements (ΔH) for turbidity formation were not related directly to any one of these factors. In cases where the linear relationship was "bent," there may be a nonequilibrium situation with particles coalescing in preference to formation of new particles, and the consequent effect on turbidity could change the temperature dependence of the colloid's equilibrium constant.

Guanidine hydrochloride (1 and 6 M) was an effective inhibitor of cold-dependent turbidity, effectively maintaining clear parotid saliva at 2°. Comparable concentrations of urea were not as effective in inhibiting colloid formation, although urea did produce significant reductions in turbidity. Guanidine hydrochloride is thought to break down hydrophobic interactions and hence denature proteins and separate subunits, whereas urea has been found to enhance hydrophobic bonding (24), the reason for its denaturating effect being unknown. The hydrophobic bonding of the colloid in parotid saliva is probably destroyed by guanidine hydrochloride, resulting in disaggregation of the proteins, whereas urea, because of its polar nature, may only destroy the ionic or hydrophilic bonding of proteins and phosphate ions causing less drastic destabilization of the aggregates. Since pellet protein and pellet phosphate concentrations correlated, phosphate ions may bind specifically to colloidal aggregates, either stabilizing the surface of particles or forming electrostatic bonds between protein in the aggregates.

The colloidal aggregates may have a partial ordered arrangement of proteins ($\Delta S < 0$) possibly because of (*1*) regions of hydrophobic bonding between nonpolar portions of proteins, (*2*) phosphate binding at cationic sites on proteins, and (*3*) the surface orientation of hydrophilic groups of proteins.

The protein composition of parotid colloid has been partially characterized (2), showing preferential involvement of some parotid proteins, in particular "proline-rich" proteins and a phosphoprotein.

Descriptions of protein micelles or colloid in physiologic fluids are rare. The best described protein micelle system is the casein micelle (22, 23, 31), although recently a cold-dependent turbidity has been found in boar seminal plasma (10, 16) (Table 5) and in human pancreatic juice (1).

The white opalescence characteristic of milk can be attributed to light scattering from small spherical micelles of casein (0.04–0.30 μm diameter). Milk micelles do not form at 0°–6°, but form preferentially at 37° and require calcium (31) (Table 5).

Proteins in boar seminal plasma form a zinc-stabilized cold-

Table 5. *Comparison of physicochemical requirements for formation of micelles in milk, zinc-promoted opalescence in boar seminal plasma, and colloid formation in parotid saliva*

Milk (22, 23, 31)	Boar seminal plasma (10, 26)	Parotid saliva
1. Micelles form at 37°; no formation at 0°–6°	1. Colloid opalescence forms at 4°; clears at 37°	1. Colloidal turbidity increases on cooling to 2°; clears at 37°
2. Critical minimum amount of calcium essential for stability	2. Zinc is essential for stability of turbidity; EDTA and citrate clear turbidity	2. Calcium is not essential for turbidity formation; EDTA does not effect cold-dependent turbidity; guanidine hydrochloride and urea clear turbidity
3. Micelles contain calcium, α_{s}-, β-, and κ-caseins bound in a stable structure	3. Colloid consists of protein of molecular weight approx. 50,000 which binds zinc	3. Colloidal aggregates consist of one or more parotid proteins and bound phosphate ions

dependent turbidity (10, 26) (Table 5). The turbidity is present as an opalescence at 37° and increases on cooling, behaving in a reversible manner similar to parotid saliva, but being inhibited by chelation of zinc with EDTA. Turbidity in the cold increases with increasing zinc content and decreases with increasing EDTA or citrate concentrations, but is independent of calcium.

Thus, the colloid formation of parotid saliva is cold induced and independent of divalent cations, that of milk has reversed temperature requirements and a calcium dependence, and that of seminal plasma is cold induced and zinc dependent (Table 5).

It is of interest that in CF seminal plasma higher concentrations of zinc, calcium, magnesium, and enzymes and decreased solubility of protein have been reported (27). Adult male CF patients are usually sterile, probably because of atrophy of the vas deferens subsequent to blockage by insoluble material (25).

In postoperative human ductal pancreatic juice, two types of protein precipitation have been observed (1). Protein concentration-dependent fine precipitates formed on chilling inactive pancreatic juice and redissolved on rewarming to 37°. In activated pancreatic juice, flocculent precipitates were formed, which did not redissolve at 37°. Cation dependence was not investigated.

Chronic ethanol administration to dogs has shown rises in cholecystokinin-stimulated pancreatic juice protein concentration and output after 6 weeks, and falls after 12 months (30). After 6 weeks, microcalculi (rich in protein and calcium) and protein plugs were excreted from the main pancreatic duct, and it was considered that these may be causing incipient pancreatic lesions because of an obstructive mechanism, thus leading after 12 months to reduced pancreatic secretion. A similar type of obstructive mechanism may be operative in the pancreas of the cystic fibrosis child.

CRYSTAL FORMATION IN PAROTID SALIVA

At 37°, parotid saliva samples often showed a gradual increase in turbidity, and analyses of centrifuged pellets indicated formation of insoluble calcium, phosphate, and protein.

Analysis of 2-hr 37° turbidity with transmission electron microscopy and electron diffraction has shown crystalline material which was often identified as hydroxyapatite (2). However the pellet Ca/P ratio of 0.97 ± 0.34 and the presence of amorphous material by electron microscopy suggests incomplete crystallization at 2 hr. Change of pH because of loss of carbon dioxide probably contributed to formation of insoluble calcium phosphates in saliva samples with sufficiently high Ca/P products to cause precipitation (29).

Grøn (17, 18) found stimulated parotid saliva from normal subjects to be saturated in respect to hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), whitlockite ($Ca_3(PO_4)_2$), and octacalcium phosphate ($Ca_4H(PO_4)_3$). The calculations were based on ionized calcium and ionized orthophosphate determinations. The tendency toward higher calcium concentrations in parotid saliva of children with cystic fibrosis (4, 6) would induce a greater degree of hydroxyapatite crystal and turbidity formation in their saliva.

The protein bound to the calcium-phosphate complex has been characterized (2) as a phosphoprotein (9) and as proline-rich proteins (3). It was noted that the presence of enough EDTA to chelate all the diavalent ions resulted in no precipitation of the calcium-phosphate-protein complex at 37° (suggesting that protein was adsorbed to the mineral (19)), yet allowed the formation at 2° of the colloid containing the same proteins. Urea and guanidine hydrochloride also diminished turbidity formation at 37°.

The calcium-phosphate-protein complex formed in parotid saliva with calcium concentrations exceeding 0.5 mM both in CF patients and non-CF subjects. Similar formation of hydroxyapatite was noted in CF submandibular saliva after standing at room temperature for 2 hr (5, 8) and in whole saliva of heavy calculus formers (11) after 20 hr of incubation at 37°. Electrophoretically the turbid material of the CF submandibular saliva contained protein bands postulated to be derived from undissolved components of zymogen granules (5). CF children have an increased incidence and severity of dental calculus on tooth surfaces adjacent to the ducts of both the submandibular-sublingual glands and the parotid glands (32).

RELEVANCE TO OBSTRUCTION BY EXOCRINE SECRETIONS IN CF

It has been suggested that the insoluble material of CF submandibular saliva, i.e., undissolved "spherules" (zymogen granule components) and hydroxyapatite crystals, could block the ducts of the submandibular gland (5). Similarly the material formed at 37° in this study, i.e., hydroxyapatite crystals and associated protein, may obstruct the ducts of the parotid gland (20, 28). Elevated concentrations of protein and calcium in CF compared with control parotid saliva (4, 6) would indicate a higher likelihood of duct blockage in CF patients, since turbidity increases more rapidly at higher calcium levels, as does the amount of insoluble complex formed. Unstimulated resting parotid saliva has high calcium and protein levels (13, 14). It seems feasible that overnight in unstimulated glands of the CF child there may be deposition of the calcium-phosphate-protein complex in stagnant saliva in parotid acini and ducts. In both CF and non-CF subjects, parotid calculi and dental calculus on teeth may have a similar origin.

SUMMARY

Two processes of turbidity formation in parotid saliva have been described in this study. Colloidal aggregates of proteins formed at temperatures below 37° in increasing amounts related to increasing protein concentration and decreasing temperature. The formation of colloid was reversible (clearing instantaneously at 37°), independent of the presence of divalent ions, exothermic in nature, and resulted in a more ordered arrangement of proteins in the saliva. The colloidal particles (confirmed by electron microscopy (2)) consisted of several proteins (characterized by electrophoresis (2)), and some specifically bound phosphate ions. There was relatively little cold turbidity in the presence of urea or guanidine hydrochloride.

The second type of turbidity consisted of a calcium-phosphate-protein complex which formed most rapidly at 37°, and more slowly at lower temperatures. Turbidity, and consequently the amount of calcium, inorganic phosphate, and protein deposited in the pellet after incubation at 37°, increased with increasing total calcium concentration. Protein in the pellet also increased proportionally to total protein concentration. The addition of EDTA to parotid saliva in amounts sufficient to chelate all divalent ions inhibited the formation of the 37° turbidity. The crystalline material contained hydroxyapatite (identified by electron microscopy (2)) and proteins (characterized by electrophoresis (2)).

No qualitative differences were observed between turbidity formation in parotid saliva of children with cystic fibrosis and the young adult non-CF subjects of this study. However, the positive correlations between cold-dependent turbidity and protein concentration, and between 37° turbidity and calcium concentration, indicate a greater degree of turbidity formation in parotid saliva of CF children. This quantitative difference is consistent with an increased incidence and severity of parotid small duct obstruction and dental calculus adjacent to Stenson's duct in children with cystic fibrosis.

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Colloid
crystals
cystic fibrosis
electrophoresis
parotid saliva
phosphoprotein

Colloid and Crystal Formation in Parotid Saliva of Cystic Fibrosis Patients and Non-Cystic Fibrosis Subjects. II. Electron Microscopy and Electrophoresis

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Extract

Centrifuged pellets of turbid parotid saliva from cystic fibrosis (CF) patients and non-CF subjects, obtained from saliva kept at 2° for 10 min, had the electron microscope appearance of amorphous, round particles, and were thought to be colloidal aggregates of organic material. Drops of turbid saliva, from samples incubated for 2 hr at 2° or 37°, additionally contained discrete, electron-dense crystals having well defined angular morphology: usually cubic, rectangular, or approximately hexagonal. The inhibitors, urea, guanidine hydrochloride, and EDTA, resulted in no crystals being observed. Selected area electron diffraction from individual crystals showed predominantly hexagonal, rectangular, or square diffraction patterns. The hexagonal and rectangular patterns could be indexed as coming from hydroxyapatite. A transition from the hexagonal to the rectangular pattern and back to the hexagonal pattern could be obtained from individual crystals tilted in the electron microscope. The square diffraction pattern may be from octacalcium phosphate or brushite.

Polyacrylamide gel disc electrophoresis of the parotid saliva in-