that in paper electrophoresis, chylomicrons remained around the starting point while lipomicrons migrated with the beta-globulins. Since lipomicrons would probably be denatured by lyophilization it is possible that the increase in the cholesterol remaining around the starting point in the lyophilized sera may represent denatured lipomicrons.

The results which have been presented suggest that the cholesterol which is extracted by cold chloroform from lyophilized serum is primarily that present as a constituent of lipo- and chylomicrons. Additional work, however, is necessary to establish this. It is interesting that Albrink, Man and Peters(11) found that the neutral fat which was removed by centrifugation at 18,000 rpm for 1 hour from clear and lactescent sera having total cholesterol values above 300 mg per 100 ml varied from 16.8 to 478 mEq per liter while the amount remaining in the subnatant varied only from 10 to 34 mEq per liter. They assume that the lipids which rose to the surface ranged from 0.1 μ in diameter, the size just sufficient to scatter light(12) to 1 μ, the size of larger chylomicrons. Any cholesterol associated with this material undoubtedly would be included in our REC fraction.

Summary. Lyophilization of serum has been shown markedly to retard electrophoretic migration of some of the cholesterol normally associated with the beta-globulin area but to exert little effect on the cholesterol associated with the albumin + alpha$_1$-globulins. The cholesterol which is extracted by cold chloroform from lyophilized serum comes primarily from that fraction normally found in the beta-globulin area or which remained around the starting point. It is suggested that the cholesterol thus removed represents primarily cholesterol which is associated with lipo- and chylomicrons.

1. Forbes, J. C., Dillard, G. H. L., Porter, W. B., and Petterson, O. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 240.

2. Forbes, J. C., Camp, P. D., Jordon, W. R., Petterson, O. M., and Korn, C., *South. Med. J.*, 1954, v47, 226.

3. Forbes, J. C., *Am. Pract. Dig. of Treat.*, 1955, v6, 907.

4. Köiw, E., Wallenius, G., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 47.

5. Forbes, J. C., and Taylor, P. C., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 411.

6. ———, *Fed. Proc.*, 1954, v13, 209.

7. Hunter, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 538.

8. Oliver, M. F., and Boyd, G. S., *Brit. Heart J.*, 1955, v17, 299.

9. Gurd, F. R. N., Oncley, J. L., Edsall, J. T., and Cohn, E. J., *Discussions Faraday Soc.*, 1949, v6, 70.

10. Swahn, B., *Scand. J. Clin. and Lab. Invest.*, 1953, v5, Suppl. 9.

11. Albrink, M. J., Man, E. B., and Peters, J. P., *J. Clin. Invest.*, 1955, v34, 147.

12. Aherns, E. H., Jr., and Kunkel, H. G., *J. Exp. Med.*, 1949, v90, 409.

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Coagulation of Salivary Mucoid by Freezing and Thawing of Saliva.* (22329)

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Salivary mucoid,† especially when freshly obtained, may be readily precipitated in the form of a coagulum by the addition of dilute acid to the mucoid-containing fluid(2-4). This characteristic has been used to identify and to prepare mucoid of salivary gland origin(2-5).

It has now been observed that freezing of chemically untreated mucoid-containing salivary secretions for varied periods of time re-

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† The terminology suggested by Meyer(1) is used throughout this report.

sults in the formation of a persisting coagulum when the secretion is thawed. This coagulum, or clot, generally resembles in appearance the mucin clot formed in response to the addition of acid. Evidence from viscosity measurements, response to dilute acetic acid, and analyses of "sialic acid" indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid.

Methods. Total saliva and the separate salivary secretions were collected by procedures described previously(6,7). *Chemical Methods.* The mucin clot test for the presence of polymerized mucoid was performed by adding 2 volumes of 3% acetic acid to one volume of saliva. "Sialic acid", which appears to represent the principal constituent of the carbohydrate moiety of the mucoid(4), was detected by the direct Ehrlich reaction according to the method of Werner and Odin (8). Since for this investigation only the direction of change in "sialic acid" concentration resulting from removal of the clot following freeze-thaw treatment is of importance, only relative concentrations of "sialic acid" (in terms of optical densities measured at 5650 Å in the Coleman spectrophotometer) are indicated. For amylase determinations, salivary samples were diluted 1:1000 with .025 *N* sodium chloride buffered to pH 6.9 with .01 *M* sodium phosphate. The reaction mixture consisted of 1.0 ml buffered .025 *N* sodium chloride, 1.0 ml stock soluble starch solution(9), and 0.5 ml diluted saliva. Digestion was for 15 minutes at 37.0°C. Enzyme activity is expressed in terms of milligrams of reducing sugar x 10 formed from 10 mg of substrate under these conditions. The method of Myers, Free, and Rosinski(9) was used for determination of reducing sugar. *Physical methods.* Freezing of saliva samples was accomplished either by immersion of a pyrex container in a dry ice-acetone mixture at −60 to −70°C or by placing at −10°C in the freezing compartment of a refrigerator. In each case the method and duration of freezing are stated. Thawing took place at room temperature (22-25°C) in all cases. Saliva it not a true Newtonian fluid; consequently the rate of flow of salivary fluid through a narrow bore tube in response to a difference in pressure may, to some degree, reflect the influence of physical characteristics other than viscosity. Nonetheless, it is generally true that this resistance to flow results principally from the presence of polymerized salivary mucoid and decreases when the mucoid is depolymerized(10,11) or removed as by acid clotting. With the reservations imposed by the nature of the salivary fluid clearly recognized, therefore, viscosity measurements may be used to reflect grossly the concentration of polymerized salivary mucoid(11,12). In this investigation, viscosity measurements were made using graduated capillary pipettes held vertical in matched test tubes containing 2 ml of salivary fluid. The pipettes were calibrated by observing emptying times for a 40% sucrose solution.

Results. The appearance of a persisting clot in fresh samples of frozen and thawed, but otherwise untreated, total mixed, submaxillary and sublingual salivas has been observed using more than 200 secretion samples from at least 40 individual subjects. The clot appears in the mucoid-containing secretions within 2 minutes if dry ice is employed for the freezing stage. That the clot formed after thawing is derived from the mucoid is indicated by observations resulting from 3 types of experiments. In the first type, dilute acetic acid was added to an untreated aliquot of a submaxillary secretion sample and to the supernatant fluid of a frozen-thawed aliquot at the same time after collection of the sample. Fig. 1 shows clearly the mucin clot which formed in the control and that no clot or precipitate appeared in the supernatant of the frozen-thawed aliquot.

The viscosity of a sample of submaxillary saliva standing at room temperature (25°C) may decrease by 50% within 40 minutes after collection (Table I). This effect has been attributed to nonenzymatic(10) and enzymatic(13) depolymerization processes. Freezing and thawing of an aliquot of the submaxillary saliva sample followed by removal of the resulting clot leads to an even more marked reduction of viscosity than is observed for the control aliquot within the same time

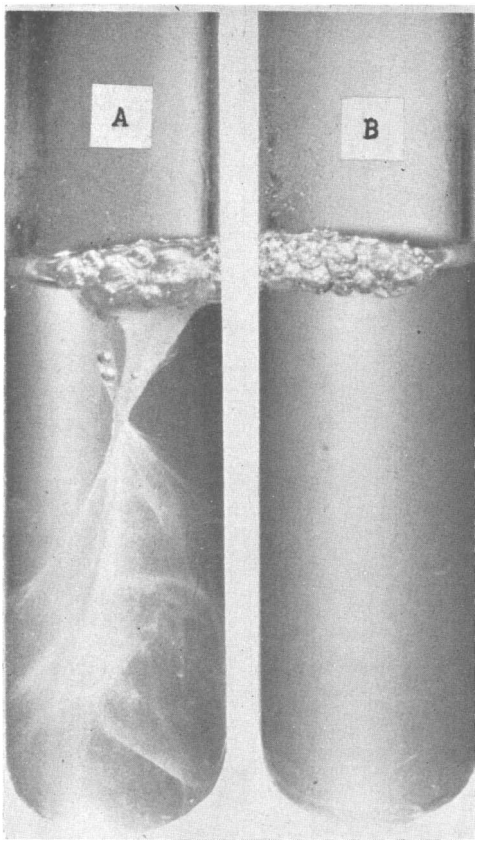

FIG. 1. Effect of addition of dilute acetic acid to an untreated aliquot (A) and to supernatant fluid of a frozen-thawed aliquot (B) of a submaxillary secretion sample 40 min. after collection. Note absence of acid-induced clot in (B), indicating decrease in polymerized mucoid in supernatant after freezing at −60°C for 2 min. and subsequent thawing at 25°C.

period after collection and indicates significant added loss of polymerized mucoid from solution as a result of the freeze-thaw treatment (Table I).

A third line of investigation which indicates that the clot which appears following thawing is derived from mucoid consists of comparing "sialic acid" values of sample aliquots which are untreated and those from which the clot appearing after freezing and thawing has been removed. "Sialic acid" is a major constituent of submaxillary mucoid and represents 25-30% of its dry weight(4). As shown by the data given in Table II, a marked decrease in "sialic acid" occurs in those aliquots submitted to freezing and thawing followed by

TABLE I. Effect of Freezing and Thawing on Viscosity of Submaxillary Secretion Samples.

	Sample I	Sample II
	Centipoises	
Initial viscosity	9.2	13.4
Final viscosity* (no freeze-thaw)	6.7	6.6
Final viscosity* (after freeze-thaw†)	2.4	1.8

* Final viscosity was determined 40 min. after initial viscosity determination. Except for period of freezing (where indicated), aliquots were kept at 25°C.

† Frozen by immersion in dry ice-acetone mixture at −60°C for approximately 2 min., thawed at 25°C, centrifuged, and clot discarded.

removal of the resulting clot (Table II).

It may be noted also that clot formation after freezing and thawing is not observed with parotid secretion samples. The "sialic acid" analysis for a parotid secretion sample noted in Table II suggests the absence of "sialic acid" from this secretion. These observations are consistent and further indicate that the clot resulting from freezing and thawing of saliva samples is derived from the salivary mucoid.

TABLE II. Effect of Freezing and Thawing on Relative Concentration of Sialic Acid in Salivary Secretion Fluids.

Subject	Sample	Aliquot I (untreated)*	Aliquot II (frozen-thawed)
		Optical density × 10^2	
HF†	Submaxillary	14	5
	"	14	5
FB‡	Parotid	0	
	Sublingual	34	
	Submaxillary	17	7
LS§	"	15	3
LL§	"	43	7

* Untreated aliquots kept at 25°C before analysis.

† Aliquots I and II were submitted to analysis simultaneously 40 min. after collection of sample. Before analysis, Aliquots II were frozen 2 min. in dry ice-acetone mixture, thawed at room temperature, centrifuged and the clots discarded.

‡ Aliquots I were submitted to analysis within 30 min. after sample collection. Aliquot II (submaxillary sample) was submitted to analysis after freezing for 1 hr at −10°C and thawing at room temperature.

§ Aliquot I was submitted to analysis immediately after collection of the sample. Aliquot II was frozen 24 hr at −10°C and thawed at room temperature before analysis.

The extent to which the concentration of substances, other than mucoid, may be modified in the salivary fluid by inclusion in the coagulum has been estimated at this time only for the protein enzyme amylase. The data in Table III indicate that the amylase concentration of the salivary fluid is not appreciably modified either by the freezing and thawing procedure or by removal of the clot resulting from this procedure (Table III).

TABLE III. Effect of Freezing and Thawing of Saliva Samples on Their Amylase Activity, 5 subjects.

	Amylase activity*			
	Unfrozen aliquot		Frozen-thawed aliquot	
Secretion	Immed. after collection	After 24 hr at 4°C	Clot removed	Clot resuspended
Submaxillary	2.2	2.2	2.3	2.5
Parotid	7.9	7.9	7.9	
Submaxillary	6.8	6.4	6.2	5.4
Parotid	13.9	12.8	12.3	
Submaxillary			3.8	4.7
Total mixed			2.7	2.7
Idem			2.8	3.1

* Conditions: Amylase activity is expressed as mg reducing sugar × 10 formed at 37°C from 10 mg of starch by the action of .5 ml 1:1000 saliva. Freezing (where indicated) was for 24 hr at −10°C in refrigerator; thawing occurred at room temperature (22-25°C).

Discussion. Gore has reported that stimulated total or mandibular (mixed submaxillary-sublingual) saliva shows some opacity at collection and that on standing at 37°C for one hour appreciable precipitate, presumably of mucoid material, appears and settles out (14-16). This effect may possibly be ascribed to the action of a contaminating mucinase (12,13). The separate submaxillary secretion obtained for the experiments reported here not only has shown no opacity on collection but generally shows only slight turbidity even after standing for two hours at 25 or 37°C. For the clear delineation of the freeze-thaw effect, therefore, separate submaxillary secretion has generally been used.

The observation that a persisting clot appears after thawing of frozen fresh, mucoid-containing saliva samples and the experimental data which show clearly that this clot is derived from salivary mucoid may have application from several points of view. First, it is customary and convenient to freeze tissue or fluid samples for storage before analysis. In the case of saliva samples, care must be taken to determine that such treatment does not alter the composition relative to the analysis in question. Second, freezing and thawing may provide a convenient method for reducing the viscosity of the mucus gland secretions especially in cases where this does not alter the concentration of other components. In addition, the freeze-thaw method may prove useful as a gentle procedure for the preparation of mucoid. This method has been used successfully for the preparation of fibrinogen of extreme purity(17). The purity of salivary mucoid separated by freezing and thawing has not been determined, particularly because of difficulties involved in establishing adequate criteria of purity(1). Finally, a difference in physico-chemical properties of salivary mucoid and other mucus secretions is indicated, since Ronkin(18) has reported no change in properties after thawing frozen hyperbranchial mucus secretion from the marine snail, *Busycon*.

Summary. 1. Freezing of chemically untreated mucoid-containing salivary secretions results in the formation of a persisting clot when the secretion is thawed. This clot resembles in appearance the mucin clot formed in response to the addition of acid. 2. Evidence from viscosity measurements, response to addition of weak acid, and "sialic acid" analyses indicates that the coagulum formed after freezing and thawing is derived from the salivary mucoid. 3. It is suggested that this effect be taken into account if storage of saliva samples in a frozen state is considered.

1. Meyer, K., *Advances in Protein Chem.*, 1945, vII, 249.

2. Obolensky, S., *Pflugers Arch. ges. Physiol.*, 1871, v4, 336.

3. Hammarsten, O., *Z. physiol. Chem.*, 1888, v12, 163.

4. Blix, G., Svennerholm, L., and Werner, I., *Acta Chem. Scand.*, 1952, v6, 358.

5. Knox, K. W., and Still, J. L., *J. Dental Research*, 1953, v32, 379.

6. Schneyer, L. H., *J. Dental Research,* 1955, v34, 257.

7. Schneyer, L. H., and Levin, L. K., *J. Appl. Physiol.,* 1955, v7, 508.

8. Werner, I., and Odin, L., *Acta Soc. Med. Upsaliensis,* 1952, v57, 230.

9. Myers, V. C., Free, A. H., and Rosinski, E. E., *J. Biol. Chem.,* 1944, v154, 39.

10. Ericsson, Y., and Stjernstrom, L., *Oral Surg., Oral Med., Oral Pathol.,* 1951, v4, 1465.

11. Dewar, M. R., and Parfitt, G. J., *J. Dental Research,* 1954, v33, 596.

12. Robertson, W. V. B., Ropes, M. W., and Bauer, W., *J. Biol. Chem.,* 1940, v133, 261.

13. Knox, K. W., *J. Dental Research,* 1953, v32, 367.

14. Gore, J. T., *Dental Cosmos,* 1935, v77, 942.

15. ———, *J. Dental Research,* 1938, v17, 69.

16. ———, *ibid.,* 1956, v35, 102.

17. Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.,* 1947, v13, 231.

18. Ronkin, R. R., *Arch. Biochem. and Biophys.,* 1955, v56, 76.

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Serum Glutamic Pyruvic Transaminase in Cardiac and Hepatic Disease.* (22330)

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Characteristic alterations of serum glutamic oxaloacetic transaminase (SGO-T) activity have been shown to be useful in assessing the presence and degree of heart muscle cell and hepatocellular damage(1,2). The activities of glutamic oxaloacetic (GO-T) transaminase and of glutamic pyruvic transaminase (GP-T) in different tissue homogenates are compared in Table I. The activity of GP-T was found to be relatively greater in liver than other tissues as compared with GO-T activity. This observation suggested that serum glutamic pyruvic transaminase (SGP-T) might be a more specific index of liver cell damage than SGO-T because of its selective concentration in liver tissue. Furthermore, since the cardiac tissue activity of GP-T is low, myocardial necrosis might not be associated with significant alterations of SGP-T activity, thus providing a more specific serum enzyme indicator of hepatocellular damage.

Method of study. The SGP-T activity was measured spectrophotometrically. The transamination reaction (Reaction 1) is coupled to the reduction of pyruvate to lactate by reduced diphosphopyridinenucleotide (DPNH), in the presence of added excess of purified lactic dehydrogenase (Reaction 2).

$$\alpha\text{-keto glutarate} + \text{l-alanine} \xrightarrow{\text{SGP-T}} \text{glutamate} + \text{pyruvate} \quad \text{(Reaction 1)}$$

$$\text{pyruvate} + \text{DPNH} \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{lactate} + \text{DPN} \quad (\text{ " } 2)$$

Oxidation of DPNH, and thereby the transamination reaction, is followed by measuring the decrease in light absorption at wavelength 340 mμ at which reduced diphosphopyridinenucleotide has an absorption peak. The *reagents* include: alanine, α-keto-glutaric acid, reduced diphosphopyridinenucleotide, and purified lactic dehydrogenase which can be obtained commercially. From 0.1 to 1.0 ml of serum, 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 0.5 ml of 0.2 M l-alanine in buffer (pH 7.4), 0.2 ml of DPNH (1 mg/ml) and 0.1 ml of a solution of purified lactic dehydrogenase (8000 units/1.0 ml) were mixed and brought to final volume of 2.8 ml in a cuvette having a 1 cm light path. The blank contained all reactants listed except DPNH. After 15 minutes, 0.2 ml of 0.1 M α-ketoglutarate in buffer, pH 7.4, was added. The optical density at wavelength 340 mμ was

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