E. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Rinehart stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

F. Apical portion of a molar root from a guinea pig treated with L-ascorbic acid. Alloxan-Schiff stain. Note normal morphology and staining characteristics of dentin formed during restoration period. × 73.

G. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Rinehart stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to retain the stain for acid mucopolysaccharides, whereas predentin in Plate IE does not. Dentin stains normally. × 73.

H. Apical portion of a molar tooth from a guinea pig treated with D-ascorbic acid. Alloxan-Schiff stain. Note normal morphology of dentin and predentin. Note also that predentin (arrow) tends to remain unstained, indicating lack of reactive proteins, while dentin stains normally. × 73.

redox properties can substitute. Separation of biological activities of Vit. C has been indicated by studies with D-araboascorbic acid in guinea pigs(4) and with D-ascorbic acid in monkeys(11). One of the best examples of non-specific role for the vitamin has come from studies of tyrosine metabolism in which L-ascorbic acid can be replaced by various other structurally unrelated compounds that are susceptible to oxidation and reduction(12, 13). Further studies with D-ascorbic acid in guinea pigs may throw light on the nature of non-specific functions of the vitamin.

1. Dayton, P. G., Burns, J. J., *J. Biol. Chem.,* 1958, v231, 85.

2. Burns, J. J., Dayton, P. G., Schulenberg, S., *ibid.,* 1956, v218, 15.

3. Demole, V., *Biochem. J.,* 1934, v28, 770.

4. Reichstein, T., Demole, V., *Festschrift,* Emil C. Barell, Basel, 1936, v107.

5. György, P., *Vitamin Methods,* Vol. I, Academic Press, N. Y., 1951, 244.

6. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry,* 2nd ed. Blakiston Co., N. Y., 1954.

7. Rinehart, J. F., Abul-Haj, S. K., *Arch. Path.,* 1951, v52, 189.

8. Yasuma, A., Ichikawa, T., *J. Lab. and Clin. Med.,* 1953, v41, 296.

9. Woodruff, C. W., Cherrington, M. E., Stockell, A. E., Darby, W. J., *J. Biol. Chem.,* 1949, v178, 861.

10. Solomon, L. L., Burns, J. J., King, C. G., *J. Am. Chem. Soc.,* 1952, v74, 5161.

11. May, C. D., Hamilton, A., Stewart, C. T., *Blood,* 1952, v7, 978.

12. LaDu, B. N., Jr., Greenberg, D. M., *Science,* 1953, v117, 111.

13. Knox, W. E., *Amino Acid Symposium,* 1955, W. D. McElroy and H. B. Glass, Baltimore, Md., 850.

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Use of Bromelin to Demonstrate Erythrocyte Antibodies.* (24828)

BERNARD PIROFSKY AND MELVIN E. MANGUM, JR.
(Introduced by Edwin E. Osgood)
Division of Experimental Medicine, University of Oregon Medical School, Portland

Technics to demonstrate circulating incomplete erythrocyte antibodies fall into 3 categories: (A) coating cells and suspending them in media containing anisometric molecules (albumin, etc.)(1), (B) an indirect Coombs' test(2), (C) pretreating erythrocytes with proteolytic enzymes (trypsin, papain, ficin)(3). The first technic is generally inadequate in range of activity and sensitivity. The Coombs' procedure is sensitive and possesses widest range of activity. However, a prozone effect, high expense, and the tedious nature of the test make it undesirable. Pretreating with proteolytic enzymes eliminated the prozone effect, but the tedious procedure persisted. By prior activation of papain with L-

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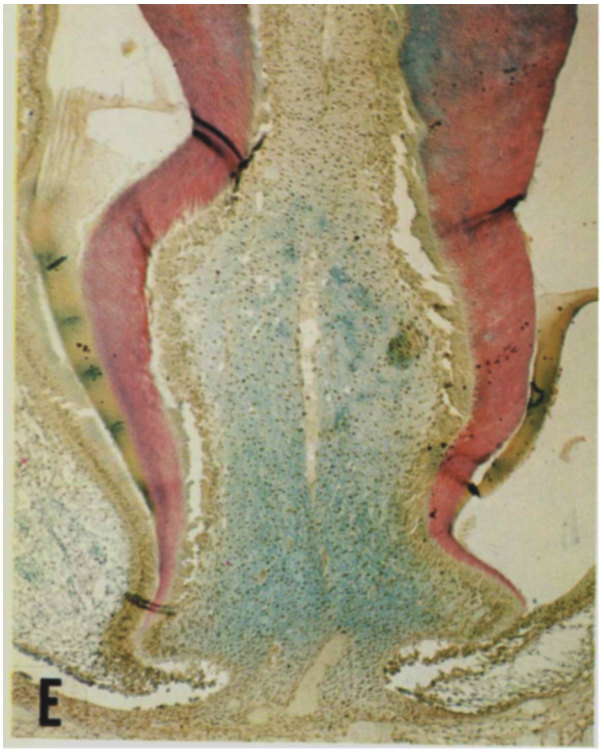
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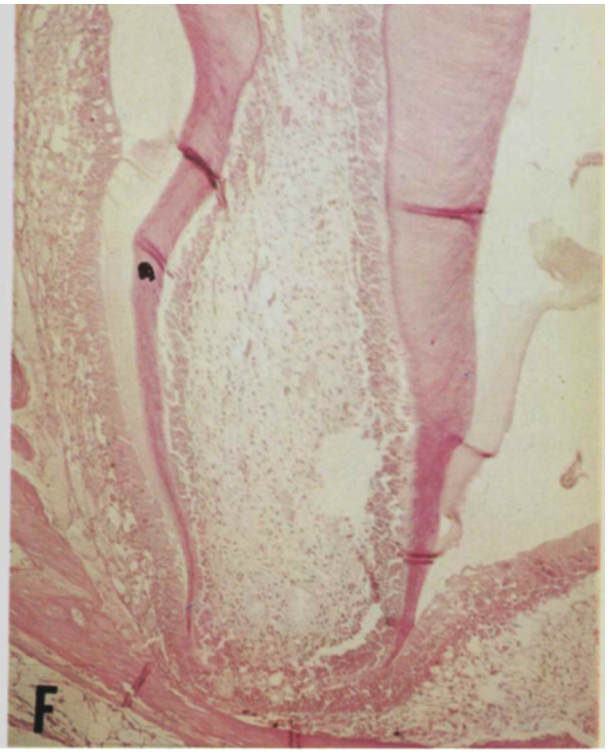
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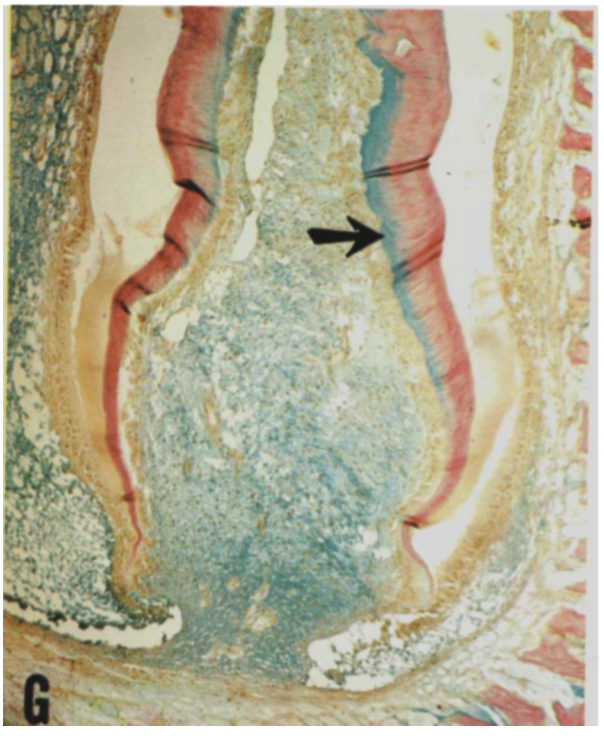
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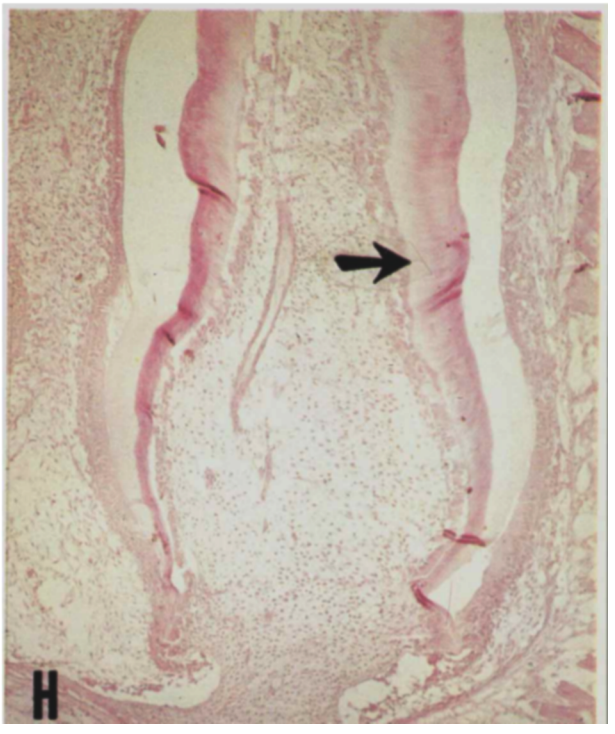
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cysteine, a technic was derived which permits direct mixing of enzyme, antibody, and target erythrocyte(4). Several problems are encountered with this method. The necessity of incubating L-cysteine with papain and storing aliquots frozen is clumsy. Marked digestion and color changes of erythrocytes may make end points difficult to read. A prolonged incubation time and sensitivity of the procedure have contributed little beyond that obtained with Coombs' technic. Major flaws in this technic are inability to demonstrate certain antibodies (MNS, Duffy, Kidd, Sutter)(5) and the lack of effect in demonstrating the incomplete antibody fixed to the erythrocyte. A new reagent and technic is now reported which possesses certain advantages over those previously used. Studies were made of a group of proteolytic enzymes derived from pineapple and supplied in a concentrated powdered form as bromelin.† Untreated isotonic solutions of this material could be mixed directly with sera containing incomplete antibodies and erythrocytes, leading to rapid, sharp, and sensitive agglutination reactions.

Methods. Various human sera containing incomplete anti-D (Rho) antibodies were serially diluted with isotonic saline. One drop of a 4% saline suspension of unwashed D (Rho) positive erythrocytes was mixed with 0.1 ml of diluted sera. One drop of bromelin solution was added directly to this mixture and macroscopic agglutination was determined after centrifugation for one minute at 1000 rpm. Optimum reaction time for this bromelin test was determined by incubating these mixtures at 37°C for varying periods prior to centrifuging. Sensitivity was determined immediately upon mixing at room temperature and after incubating for 5, 10, 15, 30, 45, and 60 minutes. An optimum bromelin reagent was determined as follows. Bromelin was dissolved in 9 parts saline and 1 part phosphate solution buffered at pH 8.6, 7.0, 6.2, 5.5, and 4.5. Concentration of the bromelin solutions was varied at 0.05%, 0.1%, 0.5% and 1%. The effect of preincubation in activating the bromelin solutions was determined by incubating solutions for one hour at 37° prior to testing. A standard bromelin test was therefore devised using unincubated reagent containing 0.5% bromelin buffered at pH 5.5. A test solution using one drop of bromelin, 0.1 ml of antibody-containing sera, and one drop of 4% saline suspension of unwashed erythrocytes was employed. Erythrocytes were chosen with an antigenic structure against which the antibody was directed. Erythrocytes not containing this antigenic group were used as controls. This mixture was immediately centrifuged one minute at 1000 rpm and inspected for macroscopic agglutination. A similar tube was incubated 15 minutes at 37°C for warm antibodies and at 4°C for cold antibodies, centrifuged and read in the same fashion. Stability of the bromelin reagent in this test procedure was determined after storage at 4°C, after storage at 4°C with 0.1% sodium azide, and after storage frozen at −20°C. Sensitivity and range of activity of these 2 bromelin procedures were compared to standard indirect Coombs' test and the activated papain-cysteine procedure as described by Stern(6). All warm-acting antibodies were incubated at 37°C and cold acting antibodies at 4°C, for one hour in Combs' and saline procedures, and for 30 minutes in the activated papain-cysteine procedure. Various human sera were tested as controls and 72 sera containing identified incomplete antibodies and 54 sera containing identified saline antibodies were investigated. Erythrocytes from 11 cases of auto-immune hemolytic anemia were studied. Each case was tested in direct Coombs' procedure and compared to various bromelin tests. A standard test was used consisting of one drop of bromelin added to 2 drops of 2% suspension of test erythrocytes. One tube was immediately centrifuged for one minute at 1000 rpm and read macroscopically for agglutination. A similar tube was incubated 15 minutes at 37°C before being centrifuged and read. The 2 test procedures were done using erythrocytes suspended in their own serum, saline suspensions of washed erythrocytes, and suspensions of washed erythrocytes resuspended in fresh, normal, ACD-collected AB plasma.

Results. Optimum reaction time for the bromelin test was 15 minutes incubation. The

† Supplied by Takamine Lab., Clifton, N. J.

immediate test, done without incubation, lowered sensitivity approximately one tube in titer studies. Similarly, increasing incubation time tended to decrease sensitivity of the reaction. Lowering the pH of the bromelin solution resulted in progressive increase of sensitivity, with optimum values obtained at pH 5.5. Increasing concentrations of bromelin progressively increased sensitivity of the reaction. Maximum values were obtained with 0.5% solutions; 1% solutions had a similar range of sensitivity. Preincubation of various bromelin solutions offered no increased titer advantages. The standard reagent used throughout remainder of this study was a 0.5% solution buffered to pH of 5.5. This solution maintained its potency approximately one month when stored at 4°C. By adding 0.1% sodium azide to the reagent, potency was maintained for over 2 months, when stored at 4°C.

Table I summarizes representative titer studies of incomplete and saline antibodies tested in the 4 standard procedures. In general, the following was noted: The indirect Coombs' procedure was frequently the least sensitive, with the papain-cysteine test usually increasing the titer by one tube. The 15-minute bromelin test often increased titers by

TABLE I. Sensitivity of Bromelin Immediate, Bromelin 15 Min., Papain-Cysteine, and Indirect Coombs' Tests with Incomplete and Saline Antibodies.

Antibody	Test titers: Bromelin, immed.	Bromelin, 15 min.	Papain-cysteine	Coombs'
Incomplete antibody				
Anti-D	1:512	1:1024	1:256	1:128
"	1:64	1:64	1:8	"
-E	1:32	1:128	1:16	1:8
-c	1:64	"	1:32	1:16
-K	"	"	1:16	1:8
-S	1:16	1:64	0	"
Saline antibody				Saline
Anti-A*	1:16	1:128	1:16	1:16
-B*	1:32	1:256	"	1:8
-D	1:256	1:512	1:256	1:64
-C	1:1024	1:2048	1:64	"
-P*	1:8	1:32	1:16	1:4
-Le^a*	1:4	1:8	1:2	1:2
-Tj^a*	1:32	1:128	1:32	1:32

* Cold acting antibodies. All tests performed at 4°C except the immediate bromelin procedure.

TABLE II. Positive Reactions of Incomplete and Saline Antibodies in An Immediate and 15-Min. Bromelin Test, a Papain-Cysteine Procedure, and the Indirect Coombs' Test.

Antibody	No.	Tests: Bromelin Immed.	Bromelin 15 min.	Papain-cysteine	Coombs'
Normal sera	600	0	0		
Incomplete antibodies					
Anti-D	35	35	35	35	35
-C	1	1	1	1	1
-E	2	2	2	2	2
-c	4	4	4	4	4
-e	2	2	2	2	2
-V	1	1	1	0	1
-S	2	2	2	0	2
-U	1	1	1	0	1
-K	8	8	8	8	8
-k	2	2	2	2	2
-Fy^a	9	7*	7*	0	9
-Jk^a	3	3	3	0	3
-Vel	1	1	1	1	1
-Js^a	"	0*	0*	0	"
Saline antibodies					Saline
Anti-A†	17	17	17	17	17
-B†	"	"	"	"	"
-D	8	8	8	8	8
-C	2	2	2	2	2
-E	1	1	1	1	1
-Le^a†	4	4	4	4	4
-P†	"	"	"	"	"
-Tj^a†	1	1	1	1	1

* Negative reactions were converted into positive reactions by using plasma suspended erythrocytes.
† All tests except the bromelin-immed. were performed after incubation at 4°C.

2 to 4 tubes over the indirect Coombs' test. The immediate bromelin test lowered titers, but was usually more sensitive than the indirect Coombs' and papain-cysteine procedures. Of particular interest is the sensitivity of the immediate bromelin test, performed at room temperature, in demonstrating cold-acting antibodies.

Table II summarizes results of testing normal sera and sera containing identified incomplete or saline antibodies by the 4 standard procedures. Six hundred normal sera were tested in the 2 bromelin procedures without a false positive reaction. In addition, no false reactions were encountered with the negative erythrocyte controls, indicating specificity of the reaction. Seventeen sera contained an antibody identified by the Coombs' procedure and not demonstrated by the papain-cysteine procedure. With the 2 bromelin tests, identi-

TABLE III. Agglutination Reactions of Auto-Antibody Coated Erythrocytes in Acquired Hemolytic Anemia; Effect of Washing the Cells and Resuspension in Normal Plasma.

	Tests			
		Bromelin, 15 min.		
Disease	Coombs'	Unwashed	Washed	Re-suspended
SLE	2+	2+	—	1+
IM	1+	1+	—	"
IHA	"	"	—	"
"	2+	3+	—	2+
"	3+	"	—	"
"	2+	4+	—	"
CLLL	3+	3+	—	"
"	2+	"	—	"
"	3+	"	—	"
"	2+	"	—	"
"	"	4+	—	"

SLE = Systemic lupus erythematosus. IM = Infectious mononucleosis. IHA = Idiopathic hemolytic anemia. CLLL = Chronic leukemic lymphocytic leukemia.

cal results were obtained; 14 of these antibodies were detected. Two of 9 anti-Fy^a and the single anti-Js^a were not detected in the standard procedure. However, when erythrocytes were suspended in fresh, normal ACD-collected AB plasma, positive reactions were obtained.

Table III summarizes use of a 15-minute bromelin test in demonstrating presence of auto-antibodies coating erythrocytes. Essentially similar results were obtained with an immediate bromelin test. When erythrocytes were unwashed and suspended in their own sera, positive results were obtained. Washing these cells and suspending in saline eliminated the bromelin reaction. Resuspending washed, coated cells in normal, fresh, ACD-collected AB plasma returned the positive reaction.

Discussion. The above studies indicate that the described bromelin test has an apparent universal application, appearing effective with incomplete, saline, warm-acting, cold-acting, natural, and immune erythrocyte antibodies. In addition, the same reagent may be used to demonstrate coating of erythrocytes by antibodies. The reagent used is stable at 4°C for at least 2 months when 0.1% sodium azide is added, and cost of the material is negligible for a standard test. The simplicity of the test makes errors in procedure very unlikely. In addition, agglutination reactions are sharp with precise macroscopic end points in titrations. The reaction is an immediate one without preincubation, washings, or incubation necessary. Sensitivity of the reaction is surprising, usually surpassing the indirect Coombs' procedure. Ability of the immediate test to detect both cold- and warm-acting antibodies eliminates the necessity of duplicate studies done at varied temperatures. The importance of such a test system in establishing a routine cross match cannot be overestimated.

An interesting phase of this reaction is still to be investigated. Three antibody-containing sera were not positive with the standard test. The 2 anti-Fy^a and the anti-Js^a which were not detected could be demonstrated when test cells were suspended in fresh ACD-collected plasma. A similar dependency on fresh plasma in this reaction was seen in demonstrating auto-antibodies coating erythrocytes. The nature of this plasma dependency is still under study.

Summary. Isotonic solutions of bromelin buffered to pH 5.5 were utilized in an immediate and 15-minute procedure to demonstrate antibodies directed against the ABO, Rh-hr, P, MNS, Kell, Duffy, Kidd, Lewis, Vel, and Sutter blood groups. In addition, a bromelin test detected erythrocytes coated with incomplete antibodies. This reaction appears to be dependent on presence of fresh plasma.

1. Jandl, J. H., Castle, W. B., *J. Lab. and Clin. Med.,* 1956, v47, 669.

2. Coombs, R. R. A., Mourart, A. E., Race, R. R., *Brit. J. Exp. Path.,* 1945, v26, 225.

3. Morton, J. A., Pickles, M. M., *Nature,* 1947, v159, 779.

4. Löw, B., *Vox Sanguinis,* 1955, v5, 94.

5. Race, R. R., Sanger, R., *Blood Groups In Man,* Thomas, 1958.

6. Stern, K., Busch, S., Buznitsky, A., *Am. J. Clin. Path.,* 1957, v27, 707.

Received February 13, 1959. P.S.E.B.M., 1959, v101.