gan to clump, and to reveal reduced growth capabilities which led to early death of the cultures. The inhibitory action of WGL on transformed cells was not destroyed by heating the preparation at 60°C, a procedure which inactivated its esterase activity for triacetin.

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1. Coman, D. R., Cancer Res., 1944, v4, 625.
2. Coman, D. R., Anderson, T. F., ibid., 1955, v15, 541.
3. Ambrose, E. J., James, A. M., Lowick, J. H. B., Nature (London), 1956, v177, 576.
4. Abercrombie, M., Heaysman, J. E. M., Korthauser, H. M., Exp. Cell Res., 1957, v13, 276.
5. Coman, D. R., Cancer Res., 1960, v20, 1202.
6. Easty, G. C., Easty, D. M., Ambrose, E. J., Exp. Cell Res., 1960, v19, 539.
7. Abercrombie, M., Ambrose, E. J., Cancer Res., 1962, v22, 525.
8. Easty, G. C., Easty, D. M., Ambrose, E. J., Exp. Cell Res., 1960, v19, 539.
9. Ambrose, E. J., Dudgeon, J. A., Easty, D. M., Easty, G. C., ibid., 1961, v24, 220.
10. Cormack, D. H., Easty, G. C., Ambrose, E. J., Nature (London), 1961, v190, 1207.
11. Cormack, D. H., Ambrose, E. J., Exp. Cell Res., 1963, v31, 566.
12. Aub, J. C., Tieslan, C., Lankester, A., Proc. Nat. Acad. Sci. (U. S.), 1963, v50, 613.
13. Vogt, P. K., Cancer Res., 1963, v23, 1519.
14. Koprowski, H., Pontén, J. A., Jensen, F., Ravdin, R. G., Moorhead, P., Saksela, E., J. Cell. Comp. Physiol., 1962, v59, 281.
15. Shein, H., Enders, J. F., Proc. Nat. Acad. Sci. (U. S.), 1962, v48, 1164.
16. Moyer, A. W., Wallace, R., Cox, H. R., J. Nat. Cancer Inst., 1964, v33, 227.
17. Hayflick, L., Moorhead, P. S., Exp. Cell Res., 1961, v25, 585.
18. Pontén, J., Jensen, F., Koprowski, H., J. Cell. Comp. Physiol., 1963, v61, 145.
19. Shein, H. M., Enders, J. F., Palmer, L., Grogan, E., Proc. Soc. Exp. Biol. and Med., 1964, v115, 618.
20. Wallace, R., ibid., 1964, v116, 990.
21. Hanks, J. H., Wallace, R. E., ibid., 1949, v71, 196.
22. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., Shannon, J. E., J. Nat. Cancer Inst., 1951, v11, 773.
23. Jensen, F., Koprowski, H., Pontén, J. A., Proc. Nat. Acad. Sci. (U.S.), 1963, v50, 343.
24. Todaro, G. J., Walman, S. R., Green, H., J. Cell. Comp. Physiol., 1963, v62, 257.
25. Jensen, F., Koprowski, H., Pagano, J., Pontén, J., Ravdin, R., J. Nat. Cancer Inst., 1964, v32, 917.
26. Gasic, G., Gasic, T., Nature (London), 1962, v196, 170.
27. Defendi, V., Gasic, G., J. Cell. Comp. Physiol., 1963, v62, 23.
28. Mowry, R. W., Lab. Invest., 1958, v7, 566.

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Association of Factors XI and XII with Blood Platelets.* (30217)

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The concept that physiological clotting occurs in close relationship to the platelet surface(1,2) has been supported by evidence that many of the coagulation factors are absorbed by platelets. This evidence has recently been reviewed(1,3,4). The degree to which the clotting factors adhere to the platelet surface varies widely. Some factor V(5, 6) and fibrinogen(6,7) persist on the platelet despite many washes, whereas factor VIII can be removed by several washes(3,5), and prothrombin as well as factors VII, IX and X are even more readily removed(3). Detailed information about the presence of factors XI and XII on platelets has been lacking. Caen and Haas(9) found that normal platelets increased the consumption of prothrombin in recalcified factor XI-deficient plasma. Gormsen(10) demonstrated that thrice-washed normal platelets corrected the

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impaired thromboplastin generation and thrombin generation of factor XII-deficient plasma, but that platelets from a patient lacking factor XII did this only after treatment with normal plasma. Iatridis *et al*(8) showed that washed platelets contained "surface factor," which might be activated XI (XIa†) or XIIa, but their methods did not permit them to distinguish between these activities. The present study, undertaken to resolve these conflicting reports, indicates that XI is a significant and persistent platelet constituent, whereas XII is readily reremoved by washing. A variable fraction of platelet XI is activated to XIa by the washing procedure.

Material and methods. Human blood from hematologically normal human donors and from a patient with severe congenital factor XI deficiency was collected into one-tenth volume of 3.8% sodium citrate. Plastic or siliconized glass surfaces were used in preparing and processing blood reagents. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by differential centrifugation at 4°C and were kept in an ice water bath until used. Studies were completed within 4 hours of venipuncture.

Washing procedure. One volume of PRP was centrifuged at 18,000 RPM for 15 minutes; the supernatant plasma was decanted, and the platelet button taken up in a volume of isotonic saline. Platelets were resuspended by gentle shaking. Samples of this, the first wash, were taken for clotting factor assay; the platelet suspensions were then centrifuged at 10,000 RPM for 15 minutes, the supernatant fluid again discarded, and the platelet buttons taken up with a volume of isotonic saline adjusted for that amount used to test clotting activity at the previous wash. The procedure was repeated to a total of 10 washings.

Methods for assay of clotting factors. Assays for factors V, VII and X were performed by diluting test material to 10% in distilled water, adding depleted substrate and recalcifying with a commercial thromboplastin-calcium suspension (Simplastin, General Diagnostics, Morris Plains, New Jersey). Substrate deficient in factor V was prepared by inactivating the factor V of normal oxalated plasma at 37°C, then exchanging the oxalate for citrate by dialysis. Plasma depleted of factors VII and X was prepared by the methods of Owren and Aas(13). Levels of factor V, VII and X were read from a curve prepared using as test material various dilutions of pooled normal plasmas. Factors XI and XII were tested for in new unused glassware by a modification of the partial thromboplastin time with the test material at 1:4 dilution. Factor XI-depleted plasma was prepared by shaking intact plasma with 15 mg of Filter-Cel celite (Johns-Manville, Lompoc, California) per ml, centrifuging and then incubating at 37° for 5 hours to permit inactivation of XIa(10). Factor XII-deficient plasma was obtained from a deficient donor through the courtesy of the American National Red Cross Research Laboratory. Dilution curves were made from each donor PPP for factors XI and XII, so that in each experiment the PPP value is indicated as 100% and platelet values expressed as per cent of the donor's PPP. Deficient substrates were stored frozen in small aliquots.

The methods for assay of XI and XII cannot distinguish between the precursor and activated forms of these coagulants. To detect whether XI or XIIa was produced during the washing process, a modification of the method of Henderson and Rapaport(14) was used. One-tenth ml factor XI (or factor XII) deficient substrate was incubated with 0.1 ml 1:100 dilution of Bell and Alton cephalin (15) for 2 minutes and 45 seconds; 0.1 ml 0.025 M $CaCl_2$ was added, followed exactly 15 seconds later by test material diluted 1:4 in imidazole buffered saline (pH 7.3). Clotting time was determined from the time of addition of the unknown. This procedure

† Increasing evidence indicates that the clotting factors circulate as inactive precursors which are activated in the course of coagulation reactions. To avoid complicated phrases, Esnouf (quoted by MacFarlane(11)) suggested that the Roman numeral alone be used to refer to the precursor factor, and the subscript "a" be employed to designate the activated coagulant. This convention will be used here.

TABLE I. Effect of Successive Washes on Factor V of Platelets.

Exp No.	PPP	PRP	Factor V content of resuspended platelets				
			Washed 1×	Washed 2×	Washed 3×	Washed 5×	Washed 10×
			%				
1	120	125	19	19	19	14	—
2	100	120	36	13	9	6	6
3	130	135	26	25	12	12	12
4	78	100	36	32	25	25	24
5	150	150	30	30	21	21	21
Avg	116	126	31	22	17	16	16

TABLE II. Effect of Successive Washes on Factor VII and X of Platelets.

Exp No.	PPP	PRP	Factor VII and X content of resuspended platelets				
			Washed 1×	Washed 2×	Washed 3×	Washed 5×	Washed 10×
			%				
1	98	96	0	0	0	0	0
2	86	78	13	0	0	0	0
3	84	92	10	0	0	0	0
4	90	78	0	0	0	0	0
5	100	101	0	0	0	0	0
Avg	96	89	5	0	0	0	0

differs from the standard tests for factors XI and XII in that clotting is carried out in siliconized tubes instead of previously unused glass tubes. Under these circumstances, clotting is not accelerated unless activated coagulants are added, and the clotting time of intact PPP and PRP are comparable to those obtained with buffer blanks. Dilutions of XIa ("Activation product" prepared by Waaler's method(16)) in intact PPP give clotting times in this test which fall along a straight line in a log-log plot. Dilutions of glass-contacted plasma in intact PPP, however, give erratic results, perhaps because such plasma contains XIIa as well as XIa. Quantitation of XIa cannot therefore be considered precise, and we report it absent when the time in the XIa test equals that of buffer blank, present in 1+ amounts when the XIa time is 1 to 10 seconds shorter than control, present in 2+ amounts when the time is more than 10 seconds but less than 30 seconds shorter, and 3+ when greater than 30 seconds shorter. XIIa is evaluated in similar semi-quantitative fashion.

Results. The content of factors XI and XII in platelets and the effect of successive saline washings on these levels were compared with platelet levels of factors V, VII and X. The latter two factors were chosen for comparison because V adheres to platelets through many washings(3,5,17), whereas VII and X wash off completely with 2 washings(3). These results are confirmed in Tables I and II.

Factor XI activity of PRP was more than twice that of PPP (Table III). With high speed centrifugation, an amount of factor XI equal to that in PPP remained with the resuspended platelets. Half of this residual platelet factor XI remained after the fifth washing, and a third persisted after the tenth washing. The amount of XIa which could be detected varied considerably; maximal XIa was usually evident after the first or second washing but small amounts persisted through the tenth washing in 8 of the 9 experiments where this activity was sought.

Factor XII was followed in only 4 experiments (Table IV), due to a scarcity of substrate plasma for assays. Slightly less factor XII activity was evident in PRP than in PPP, and separation of the platelets from the plasma left the platelets with only a small amount of XII. Subsequent washings readily removed this activity. XIIa was detected in very small amounts in only one of the three experiments in which it was tested. Clotting times obtained in assays for factors V, VII and X, XI, XIa, XII and XIIa in a repre-

TABLE III. Effect of Successive Washes on Factor XI of Platelets.

Exp No.	XIa*	PPP	PRP	Factor XI content of resuspended platelets: Washed 1×	Washed 2×	Washed 3×	Washed 5×	Washed 10×
		%						
1	3+	100	175	76	55	50	50	—
2	1+	100	200	110	98	98	78	50
3	2+	100	185	—	100	24	10	7
4	1+	100	110	80	82	49	22	17
5	2+	100	210	98	76	70	54	53
6	—	100	240	160	—	—	100	—
7	3+	100	250	80	72	76	—	—
8	3+	100	320	55	30	16	—	—
9	3+	100	280	42	30	18	—	—
10	3+	100	170	100	70	53	—	—
Avg	2+	100	213	89	62	50	52	32

* Semi-quantitative estimation of maximal degree of factor XI activation of resuspended platelets usually noted after 3 washings.

TABLE IV. Effect of Successive Washes on Factor XII of Platelets.

Exp No.	XIIa*	PPP	PRP	Factor XII content of resuspended platelets: Washed 1×	Washed 2×	Washed 3×	Washed 5×	Washed 10×
		%						
6	—	100	60	7	—	0	0	0
8	1+	100	90	11	4	0	0	0
9	0	100	90	8	2	0	0	0
11	0	100	120	12	10	0	0	0
Avg	0	100	90	10	5	0	0	0

* Semi-quantitative estimation of maximal degree of factor XII activation of resuspended platelets.

TABLE V. Clotting Times in a Typical Experiment.

	Buffer*	PPP	PRP	Resuspended platelets: Washed 1×	Washed 2×	Washed 3×	Washed 5×	Washed 10×
	Sec							
Factor V	220	21	23	37	38	45	47	48
Factor VII and X	175	20	23	68	78	98	99	99
Total factor XI	164	85	69	88	94	97	100	105
XIa	160	160	121	126	120	115	117	116
Total factor XII	255	90	80	145	151	192	222	250
XIIa	255	188	161	220	246	252	256	254

* Imidazol—buffered saline, pH 7.35.

sentative experiment are given in Table V.

The ability of platelets to adsorb factor XI from normal plasma was tested by obtaining fresh citrated blood from a patient with congenital deficiency of factor XI (reference 12, case 1). The patient's PPP had 3% of normal XI activity and his platelets, centrifuged and resuspended in saline, contained 5% of normal XI levels. The factor XI-deficient platelets were then suspended in normal PPP. The mixture, containing 110% XI, was incubated for one-half hour at 37°C, and the platelets were then subjected to successive washes in the usual manner. After the first wash, 35% XI was found with the platelet suspension; after the second wash, 20%, after the third wash, 12%, after the fourth wash, 6%, and after the fifth wash, 4%. The experiment was repeated with similar results. Thus, XI-deficient platelets were capable of

absorbing considerable XI from normal PPP, and of maintaining it through several washings.

Discussion. The results presented here point to the conclusion that factor XI is present in high concentrations in the vicinity of platelets and that significant amounts of this factor remain with platelets, despite many saline washes. Little factor XII, on the other hand, appears to be associated with the platelet. What there is appears to be readily removed by a few washes.

With 2 saline washings a variable amount of the XI present in association with platelets is converted to XIa. This small amount of active intermediate probably accounts for the corrective action noted by Iatridis *et al* (8) of twice-washed normal platelets on the prolonged clotting time of XII-deficient plasma. As these authors suggest, the presence of small amounts of activated coagulants on washed platelets probably accounts as well for the apparent partial correction of factor VIII-deficient plasma clotting times. Platelets from XII-deficient donors, when washed, failed to accelerate any of these clotting tests. In the light of our findings, their results imply that XIa does not form during the washing of XII-deficient platelets, and that activation of XI during washing must depend on the prior formation of XIIa.

The image of platelets as sponges for protein clotting factors(1) implies that all factors are absorbed from plasma and retained on washing to a comparable degree. Clearly, this simple concept is not tenable. Three clotting factors, fibrinogen, factor V and factor XI, have now been shown to have a special affinity for the platelet. Platelets lacking factors V and XI will absorb them avidly from plasma. In the case of factor V, such absorption was demonstrated with platelets stored at 37° for 110 days(18), indicating that active metabolic processes are not required for absorption. Absorption of fibrinogen from plasma has not been clearly demonstrated, but fibrinogen has been shown by immunological methods to be present in platelets and megakaryocytes(6) and in isolated platelet granules(7). Fibrinogen and factors V and XI are made by the liver and do not require vitamin K. Although it is possible that they are synthesized by megakaryocytes, it seems more likely that they enter the platelets by absorption.

The high affinity of platelets for factors V, XI and fibrinogen may be important in the reactions leading to platelet aggregation and to formation of clots in the vicinity of platelet aggregates. How these coagulants are activated and interact requires further elucidation.

Summary. Normal platelets were subjected to successive saline washes and measurements made of factors V, VII and X, XI and XII in the resuspended platelets. Factor XI behaved like factor V in remaining with the platelet through 10 successive washes. Factor XII, on the other hand, like factor VII and X, was readily removed by one or two washes. Factor XI-deficient platelets when suspended in normal plasma absorbed over 30% of the factor XI from the plasma. Absorbed factor XI was removed by 4 washes. The potential significance of those clotting factors which tightly adsorb to platelets is discussed.

1. Adelson, E., Rheingold, J. J., Crosby, W. H., Blood, 1961, v17, 767.

2. Roskam, J., Arch. Internat. Physiol., 1923, v20, 241.

3. Bounameaux, V., Rév. Franç. Études Clin. Biol., 1957, v2, 52.

4. Marcus, A. J., Zucker, M. B., The Physiology of Blood Platelets; Recent Biochemical, Morphologic and Clinical Research, Grune & Stratton, 1965.

5. Raccuglia, G., Proc. Soc. Exp. Biol. and Med., 1960, v104, 309.

6. Gokcen, M., Yunis, E., Nature, London, 1963, v200, 590.

7. Nachman, R. L., Marcus, A. J., Zucker-Franklin, D., Blood, 1964, v24, 853.

8. Iatridis, P. G., Ferguson, J. H., Iatridis, S. G., Thromb. Diath. Haemorrh., 1964, vXI, 355.

9. Caen, J., Haas, F., Rév. Franç. Études Clin. Biol., 1960, v5, 146.

10. Gormsen, G., Thromb. Diath. Haemorrh., 1962, v7, 249.

11. MacFarlane, R. M., Biggs, R., Ash, B. J., Denson, K. W. E., Brit. J. Haemat., 1964, v10, 530.

12. Horowitz, H. I., Wilcox, W. P., Fujimoto, M. M., Blood, 1963, v22, 35.

13. Owren, P. A., Aas, K., Scand. J. Clin. & Lab. Invest., 1951, v3, 301.

14. Henderson. E. S., Rapaport, S. I., J. Clin. Invest., 1962, v41, 235.
15. Bell, W. N., Alton, H. G., Nature, London, 1954, v174, 880.
16. Waaler, B., Scand. J. Clin. & Lab. Invest., 1959, v11, Suppl. 37.
17. Hjort, P., Rapaport, S. I., Owren, P. A., Blood, 1955, v10, 1139.
18. Doezy, A., Fejer, A., Thromb. Diath. Haemorrh., 1963, v9, 300.

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Acid and Salt-Soluble Collagen in Bovine Muscle.* (30218)

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Conventional methods for determination of collagen in muscle have been based on the assumption that intracellular constituents of muscle are solubilized by alkali solutions, while connective tissue collagen is insoluble. However, both dilute acid and dilute alkali soluble components of collagen have been reported(1,2), and a cold salt soluble fraction of collagen has been found which can be changed into mature collagen by warming to body temperature(3). These soluble collagens have been postulated to be metabolic precursors of insoluble mature collagens(4). Supporting reports in the literature show that soluble collagen can be altered by many factors including temperature, growth rate, nutrition, and by various endocrine preparations(5,6,7). Most studies on the soluble collagens have been with purified sources such as tendon, and cartilage. Therefore, knowledge of previously uninvestigated soluble components of collagens in bovine muscle would be beneficial for elucidating some of the chemical and physiological changes which occur in the conversion of living muscle tissue to edible meat, and for clarifying specific relationships of collagen to meat tenderness.

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Materials and methods. The fore shank muscles from 10 yearling steers were used in this study, primarily because of their high collagen content. All samples were removed within 15 minutes of dispatching and ground through 6.2 and 3.1 mm plates of an electric grinder. At 7 days post mortem, samples were taken from the *longissimus dorsi, semimembranosus* and *triceps brachii* muscles for Warner-Bratzler shear value measurements for use in correlation of tenderness with the soluble collagen fraction. A number of difficulties were encountered when existing methods were applied in collagen determination from bovine muscle tissue. The colorimetric method of Neuman and Logan(8) for hydroxyproline determination proved unreliable for muscle extracts. Two amino acids, tyrosine and tryptophan, have been reported to interfere with the colorimetric procedure(8). Tryptophan produced 0.7% as much color as hydroxyproline, but humin formation on acid hydrolysis of proteins eliminated this interference. Tyrosine, however, was not destroyed by acid hydrolysis and produced 1.5% as much color as did hydroxyproline. The recovery of an internal standard of hydroxyproline added to beef muscle extracts was never greater than 60% using this method. Tyrosine content of the salt-soluble extract was determined spectrophotometrically (Eastoe and Courts(9)), and found to contain 375 μg of tyrosine per ml. The observed interference of tyrosine on hydroxyproline in bovine muscle is shown in Table I. Although tyrosine contributed to color development in this procedure, it actually resulted in a sup-