Ethylene dichloride, technical grade, was used as a solvent to seal the lids on the boxes.

A viscid plastic-ethylene dichloride mixture was made by placing approximately 10 cc of scrap plastic in an airtight bottle and adding approximately 40 ml of ethylene dichloride. The plastic dissolved in about 24 hr.

Preparation of specimen is shown in (Fig. 1). Brains fixed in 10% formalin solution for 2 weeks were sectioned on the cutting board (masonite bottom; ¼″ guides). With firm pressure on the tissue, sections were actually cut very slightly thicker than the ¼″ guides on the board.

The plastic box (Fig. 2), was washed with detergent dissolved in water and blotted dry with Kleenex. The brain specimen was then centered in a box of suitable size. The top was sealed to the box with ethylene dichloride applied with a brush. Bull Dog Clips, used to exert pressure, were allowed to remain in place for 10 min.

A 50-ml syringe was filled with a 5% formalin solution, which was slowly injected into the box through the 1/16″ hole (Fig. 3). The hole was then sealed with plastic-ethylene dichloride mixture.

The Thromboplastic Activity of Hyaluronate[1]

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Studies of experimental and human arteriosclerosis have drawn attention to the ground substance, or interfibrillar cement, which is an important constituent of blood vessel structure. Such substances are conspicuous in arteriosclerosis and appear to be involved in its pathogenesis (*1*). This led to a detailed study of the mucoid substances in normal and sclerotic blood vessels. In a few instances it was noted that coronary artery thromboses had developed in close relationship to the exposed mucoid materials of sclerotic intimal plaques. This observation led to a study of the possible role of such substances in the mechanism of coagulation.

The sulfate-free acid mucopolysaccharides are viscous substances of high molecular weight and would appear to be ideally situated as agents concerned with the initiation of blood coagulation (*2, 3*). A mucopolysaccharide, hyaluronic acid, is richly present in the subepithelial vascular reticular tissue of the skin and mucous membranes. Many, if not all, capillaries are supported in a mucoid ground substance that is presumably hyaluronate. A similar mucoid material is present in the intima and media of arteries and veins. The exact chemical constitution of the mucoid ground substance in normal or sclerotic blood vessels is not known. We have found, however, that much of this material can be removed with testicular hyaluronidase (*3*).

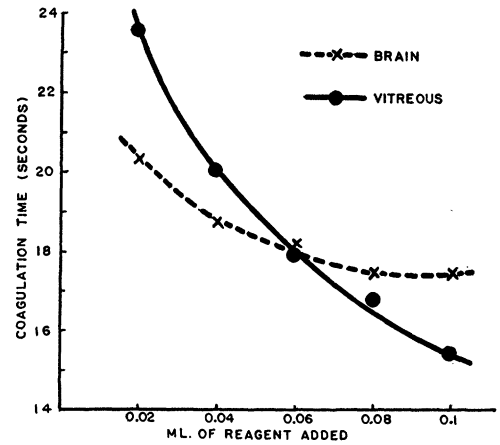

FIG. 1. Comparison of coagulation time of recalcified plasma by human brain thromboplastin and by hyaluronate.

To study the possible thromboplastic role of this mucopolysaccharide, we have used bovine vitreous humor as our source of material. The vitreous humor contains a viscous solution of hyaluronic acid, a minute quantity of an insoluble collagenlike protein ("vitrein"), soluble proteins, and small molecular substances derived from the blood plasma. The "hyaluronate" used in these experiments was the native substance of the vitreous humor removed from frozen beef eyes. After thawing at room temperature, it was centrifuged at low speed for 10 min. That portion too viscous to pipette was removed with a pair of forceps, and the remaining supernatant was utilized as a thromboplastic reagent. Further purification by the method of Meyer (*4*) or of Alburn and Williams (*5*) resulted in depolymerization and loss of thromboplastic activity. The few commercial preparations of hyaluronic acid that we have tested have not been thromboplastic. Three observations, however, cause us to attribute the thromboplastic activity of vitreous humor to the mucopolysaccharide itself: (1) The activity is not affected by centrifugation at 16,000 rpm for 2 hr, as in the method used for separating tissue thromboplastins; (2) the activity is destroyed by incubation with hyaluronidase; (3) known types of thromboplastin are not affected by hyaluronidase. It remains to be proved whether a combination of hyaluronate with native proteins is essential for thromboplastic activity.

The coagulation time of whole blood, measured by a modification of the Lee and White method at 37° C, was shortened from a control time of 12 min 40 sec to 2 min 50 sec by the addition of 0.1 ml hyaluronate, diluted with 0.9 ml water, to 1 ml fresh whole blood. This was a greater decrease than that produced by the addition of an amount of purified beef lung thrombo-

[1] This investigation was supported (in part) by research grants from the National Heart Institute of the National Institutes of Health, USPHS, and from the American Foundation for High Blood Pressure.

[2] We are indebted to Walter Seegers, of Wayne University School of Medicine, for supplying the purified prothrombin and accelerator globulin utilized in this study.

plastin equivalent to the hyaluronate activity when tested in the more purified system described below.

The activity of the hyaluronate was then compared with that of the human brain reagent as prepared by Aggeler (*6*) and used in the routine performance of the one-stage plasma prothrombin determination. In the dilution experiments illustrated in Fig. 1, a constant volume of 0.1 ml fresh plasma and 0.1 ml 0.025 *M* calcium chloride was used with varying amounts of the thromboplastin and hyaluronate, then assayed by the Quick method. The results show a greater thromboplastic activity with increasing concentrations of hyaluronate than of brain thromboplastin. Aged plasma gave slower clotting times, which could be restored to normal in the case of both thromboplastic substances by the addition of purified accelerator globulin.

For a more quantitative analysis of the thromboplastic activity of hyaluronate, we turned to the two-stage analysis described by Ware and Seegers (*7*), modified by carrying out the determinations in silicone-lined glassware at 37° C in the absence of acacia. The incubation mixture contained: (*a*) sufficient purified prothrombin to yield a maximum of 2.6 units of thrombin; (*b*) 2.5 γ/ml of purified accelerator globulin; (*c*) imidazole buffer (final pH, 7.28); and (*d*) Ca in a final concentration of 0.0112 *M*. The second stage was carried out at 37° C with 0.24% fibrinogen (Armour) in 0.9% saline. One unit of thrombin was defined as that amount which produced a clotting time of 15 sec, and was equivalent to 0.004 mg/ml thrombin (Topical, Parke, Davis & Co.). All reagents were of bovine origin.

In order to establish the fact that the thromboplastic activity of the hyaluronate was not due to contamination by tissue thromboplastin, both the vitreous humor reagent and beef lung extracts, prepared by the method of Chargaff (*8*), were preincubated with 15 mg hyaluronidase (Armour's crystalline, testicular material). As shown in Table 1, there was a marked and progressive loss of activity in the case of the hyaluronate, and no significant change in the activity of the lung extract.

The next point of interest was to determine whether calcium ions and accelerator globulin played the same

TABLE 1

EFFECT OF HYALURONIDASE ON THROMBOPLASTIC ACTIVITY OF VITREOUS HUMOR AND BEEF LUNG THROMBOPLASTIN

Time of incubation	Units of thrombin formed in first 5 min			
	Vitreous humor		Beef lung thromboplastin	
	Without hyaluronidase	With hyaluronidase	Without hyaluronidase	With hyaluronidase
1.5 hr	0.35	0.08	0.75	0.50
2.5 "	0.18	None	0.70	0.65

TABLE 2

INFLUENCE OF CALCIUM AND ACCELERATOR GLOBULIN UPON THE CONVERSION OF PROTHROMBIN TO THROMBIN BY HYALURONATE

Time of incubation	Units of thrombin formed			
	Prothrombin + hyaluronate	Prothrombin + hyaluronate + Ac-globulin	Prothrombin + hyaluronate + calcium	Prothrombin + hyaluronate + Ac-globulin + calcium
15 min	0	0	0.18	0.65
30 "	0	0	0.20	0.65

accessory roles with hyaluronate as with tissue thromboplastin. The particular demonstration illustrated in Table 2 shows that calcium is essential for hyaluronate activity, and that the rate of prothrombin conversion is considerably increased by the presence of the accelerator factor.

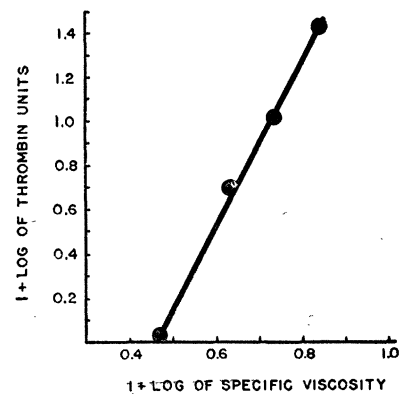

FIG. 2. Relation of log of hyaluronate viscosity to the log of thrombin yield in 5 min at 38° C. Specific viscosity = $\frac{\text{flow time (hyaluronate)}}{\text{flow time (water)}} - 1$.

From simple dilution experiments, it became obvious that the thromboplastic activity of hyaluronate was proportional to some function related to viscosity. This was studied quantitatively with the aid of a 5-ml Ostwald viscometer at a temperature of 38° C. The viscosity of various dilutions of the vitreous humor reagent was compared with that of water. Each dilution was then added to the purified system described above, and the amount of thrombin formed in the first 5 min was recorded. The log of the rate of thrombin formation was found to be directly proportional to the log of the specific viscosity, as illustrated in Fig. 2. A similar relationship between the log of plasma clotting time and the log of thromboplastin concentration has been described by Astrup (*9*).

In working with plasma, hyaluronate of fairly high viscosity must be used to demonstrate thromboplastic activity, although it is still diluted considerably more than any hyaluronate encountered in tissues. Since the

thromboplastic activity is related to the degree of polymerization of the hyaluronate, which is readily altered, resulting in loss of viscosity, the activity of low-viscosity preparations is best demonstrated in the two-stage purified system. The addition of 1% sodium chloride, a slight change of pH in either direction, successive freezing and thawing, or even standing for any length of time at room temperature are among the factors that cause both a loss in viscosity and in thromboplastic activity.

Thus it can be seen that sulfate-free hyaluronate, as it occurs naturally in the body, perhaps in combination with a protein, is an active thromboplastic agent. Its activity in coagulating blood is related to its viscosity. Like thromboplastin of tissue origin, it is dependent upon calcium, is affected by accelerator globulin, and has similar kinetics. Unlike tissue extracts, however, its activity is destroyed by hyaluronidase or by any physical procedure resulting in depolymerization. It is noteworthy that the simple sulfonation of hyaluronic acid produces a strong heparinlike anticoagulant (*10*). The widespread distribution of hyaluronic acid in tissues, and the accumulation of it or a similar substance in areas of arteriosclerosis, suggest that it may play an important role in the coagulation of blood *in vivo*.

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Tobacco Mosaic Virus Mutation

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All plant viruses that have been sufficiently studied reveal the existence of one or more strains which have evidently arisen by mutation. This subject has been frequently reviewed, most recently by Bawden (*1*). The number of strains reported for a single virus is often large, but the frequency of virus mutations has not been clearly established.

Much of the claim for high rates of virus mutation rests on the earlier studies of the yellow tobacco mosaic virus. McKinney (*2*) concluded from a study of more than 5,000 plants that the occasional yellow spots resulted not from viruses introduced from the outside, but from strains that originated as mutants in the tissues involved in the spots. Kunkel (*3*) reported that 232 plants, inoculated with 4 green strains, yielded over 5,000 yellow spots, or an average of about 6.6 spots/plant, and 0.5–3.0 spots/leaf. Jensen (*4*) and others have come to similar conclusions. Hence, the common occurrence of yellow spots on otherwise green mosaic plants has been attributed to mutations of the green virus, rather than to yellow strain contaminants in the green types.

Efforts have been made in this laboratory to determine the number of strains of the tobacco mosaic virus that are definitely determinable as such, by the available techniques. The number of these strains is found to be relatively low (*5*). If the numerous yellow variants reported to occur should be considered as mutations they would necessarily seem to be identical or recurring mutations.

To secure further information on this matter it would appear to be necessary to eliminate all possibility of contamination of green strains with yellow strains. This has been possible with improved technique as far as differentiation between green and yellow strains is concerned. The local-lesion technique in serial transfers was used to eliminate the yellow strain contamination. When green strains yield only green isolates they may yet only be assumed to be free of yellow, since more than one strain may exist in a green strain obtained from a single lesion.

Nicotiana sylvestris is well known as a test plant for yellow strains, but it unfortunately also gives local lesions with some green strains (*6*). Nevertheless, if local lesions are not obtained on *N. sylvestris* from green isolates, it appears to be satisfactory evidence that a yellow strain is not present in a totally green plant. However, local lesions on *N. sylvestris* must be verified by yellow symptoms on Havana Seed test plants to establish that a yellow strain is present if prior symptoms do not confirm this point. Tests on *N. sylvestris* often reveal the probable presence of a yellow strain long before yellow symptoms have appeared. In some instances where a single yellow spot has developed on an otherwise green mosaic plant the presence of a yellow strain has been demonstrated in areas considerably distant from the tissue involved in the spot. At least 8 strains of green mosaic have been secured that are free of yellow contamination. Many of these plants have been grown in the greenhouse, under optimum conditions for yellow strains to develop, for a period of 3 months, and to the 15–20 leaf stage without the appearance of any yellow mutations.

The reasons for easy misinterpretation of results with yellow spots are due to several circumstances, such as interference phenomena and environmental conditions. Altogether, these factors may yield an infinite variety of symptoms within fairly restricted boundaries, beyond which, however, further variation is not to be expected.

Our experimental results appear to have established the following: (1) Green and yellow strains may be readily separated where they exist in mixtures. (2) Green strains, if completely free of yellow strains, were not found to yield yellow spots. (3) Yellow areas very similar to those reported as "mutations" may be