to those of stock Bouin's. 3. Highly acidic solutions of mordanting agents in water without acetate ions do not demonstrate inclusions. Use of picric acid and water alone, and bichloride of mercury and potassium dichromate plus HCl and water demonstrated the need for acetate ions. 4. Limited experimentation with pH variations indicated that mordants with acetate ions at pH values somewhat higher than stock fixatives, are probably ineffective. Comparatively poor results obtained with Bouin's fluid at pH 5.7, and negative results with Bouin's at pH 11 and Zenker's fixative at pH 5.7, support this statement. However, Bouin's fluid at pH 11 had a high ionic strength, and was warmed to maintain solubility. 5. Acetate ions in aqueous or formalin solution at low pH without mordants is of little value. This conclusion is indicated by inability of acetic acid in water, and acetic acid in formalin to demonstrate many inclusions. 6. Aqueous solutions of mordants without acetate ions do not demonstrate inclusions. Saturated aqueous picric acid alone, and bichloride of mercury plus potassium dichromate in aqueous solution failed to reveal any inclusions. Therefore it appears that a highly acidic fixative with acetate ions and a mordanting agent is necessary for best demonstration of IBR inclusion bodies in tissue cultured cells.

Any explanation of the phenomena noted must necessarily await a more complete investigation of physical and chemical variables of the fixatives (effects of various fixation times, ionic strengths, and other substituted ions in particular), and a determination of the chemical nature of inclusion bodies. Coupled with conclusions of previous workers(1,2) this study indicates a similarity, both *in vitro* and *in vivo*, of the effects of fixatives on IBR inclusion bodies.

Summary. Primary bovine kidney epithelial cells grown in tissue culture monolayers were infected with infectious bovine rhinotracheitis virus, treated with various common fixatives, and stained with hematoxylin and eosin. Of fixatives used, Bouin's and Zenker's gave the best demonstration of characteristic intranuclear inclusion bodies. Further work with components of these 2 fluids indicated that for best demonstration of inclusions, a highly acidic fixative containing acetate ions and a mordanting agent is needed.

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Elevation of Plasma Fibrinogen Following Lymphosarcoma Tissue Injections in Rats. (24804)

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Bacterial diseases, virus diseases, trauma and cancer generally produce elevation of plasma fibrinogen level. The basic cause of the phenomenon has not been extensively investigated but most observers appear to agree with Foster and Whipple(1) that dead tissue or nonspecific (aseptic) inflammation is required to produce the elevation(2). The present paper is concerned with our observation that subcutaneous injection of cancer tissue homogenates or suspensions will cause elevation of plasma fibrinogen, whereas injection of similar preparations of a number of other tissues will not do so.

Methods. Young adult Carworth-Wistar female rats from Carworth Farms were used, and maintained on Purina dog chow and water *ad lib.* in individual cages. The Murphy-Sturm lymphosarcoma was used. It was carried by subcutaneous transplantation in the

same rat strain. To transplant the tumor, approximately 2 g of tumor tissue was pressed in a screen in 20 ml of sterile, pyrogen-free physiological saline. The tissue was sliced from peripheral regions of actively growing tumors. The tumors selected were large but had not yet reached proportions to interfere seriously with activity of the rats. The necrotic areas which occur inside of these tumors were avoided in collection of tissue for transplantation or for experimental purposes. One ml of tumor suspension in saline was injected subcutaneously to transplant the tumor. A new tumor appeared in 7 to 10 days. This grew rapidly and killed the animal in 20 to 30 days. If the tumor suspension was injected intraperitoneally, the animals developed leukemia and died in 10 to 15 days. Over 90% of all rats injected either way died from effects of lymphosarcoma. Non-viable tumor suspensions can be prepared readily by blending the mixture in a Waring Blendor for a few minutes. Homogenates of various tissues were prepared by blending them in physiological saline in the proportion of 5 g of tissue / 100 ml of saline. Erythrocytes were laked in pyrogen-free water by adding 7 ml of water to 1 ml of cells and waiting at least an hour. Plasma fibrinogen levels were determined on blood obtained by heart puncture under ether anesthesia. The assay procedure was as previously published(3), briefly 0.5 ml of blood is centrifuged; 0.1 ml of plasma is mixed with 2 ml of a thrombin solution; the clot is collected, washed in water, alcohol, and ether, then dried and weighed on microchemical balance. In some experiments fibrin was digested with sulfuric acid and hydrogen peroxide and the resulting ammonia-nitrogen determined by direct nesslerization. Amount of fibrin was then found by multiplying the ammonia-nitrogen by the factor 6.25. The 2 methods gave indistinguishable results on plasma from animals with normal or with elevated fibrinogen levels.

Results. Murphy-Sturm lymphosarcoma tissue contains a substance which causes a sharp rise in plasma fibrinogen level in rats within 24 hours after injection of tumor suspension. Curve A (Fig. 1) illustrates typical

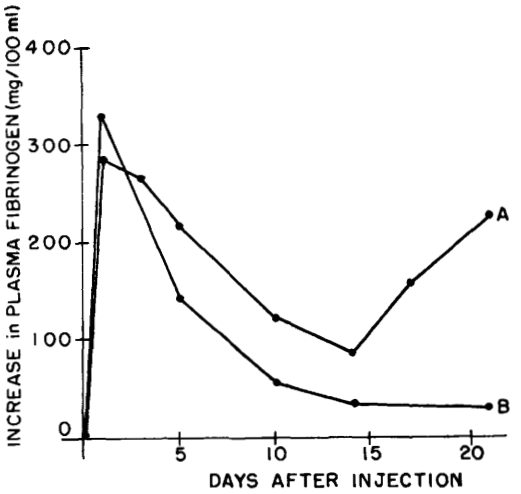

FIG. 1. Fibrinogen level following subcut. inj. of viable (A) and non-viable (B) tumor preparations.

changes in plasma fibrinogen level that accompany subcutaneous transplantation of the tumor and its subsequent growth. After the first day, the fibrinogen level drops slowly until shortly after the tumor is palpable and subsequently increases progressively as the tumor grows until the animal dies.

Tumor suspensions prepared by blending the tissue in Waring Blendor also caused a sharp rise in plasma fibrinogen level during first day after injection. Such suspensions do not produce a tumor and there was no rise in plasma fibrinogen level during third week in these rats as shown in curve B (Fig. 1).

When the tumor suspension is given intraperitoneally, the response resembles that from tumor homogenate except that the initial increase on first day is less marked. These animals, when near death, show symptoms of hemorrhage about eyes and nose and often have plasma fibrinogen levels below normal. This may result from loss of plasma internally followed by dilution of that in the vascular system.

Data in Table I show that only slight increases in plasma fibrinogen were observed after injection of homogenates of a number of normal tissues. Even liver tissue from a rat which had been refrigerated for 10 days after death (autolyzed rat liver in Table I)

caused only a modest increase. Fibrinogen assays were also run on third and fifth days after injecting these tissue homogenates, but no significant elevation was noted. The only normal tissue that caused plasma fibrinogen level to rise was the adrenal gland. However, adrenal homogenate caused extensive sloughing of skin about the point of injection, which probably accounts for prolonged elevation of fibrinogen level, extending through the fifth day.

The active principle in tumor homogenate is associated with relatively large particulates. Activity of supernatant is reduced but slightly by centrifugation at 5,000 G for 30 minutes in 8% sucrose solution, whereas centrifugation for 30 minutes at 57,000 G removed the factor.

The fibrinogen elevating factor in lymphosarcoma homogenates is stable in the physiological pH range for long periods under refrigeration and activity is retained after freezing tissue or homogenates. However, it is destroyed by heating at 100°C or by exposing it to very high or very low hydrogen-ion concentration.

Elevation of plasma fibrinogen is often associated with fever. Shear and his group have isolated a lipo-polysaccharide from tumor tissue that is quite active as a pyrogen. Dr. Shear kindly supplied us with a sample isolated from mouse adenocarcinoma 241-6 and the results of some of our tests are presented in Table II. The material caused a definite increase in plasma fibrinogen level of rats when 1-2 μg was injected intravenously/rat. This dose is of the same order of magnitude as that required for a pyrogenic or tumor-hemorrhagic response with this material(4).

TABLE I. Effect of Subcutaneous Injections of Various Tissues on the Plasma Fibrinogen Level.

Tissue	No. rats treated	Increase in plasma fibrinogen in 24 hr* (mg/100 ml)
Human erythrocytes	6	53 ± 40
Rat "	6	100 ± 40
" plasma	3	42 ± 36
Autolyzed rat liver homogenate	5	47 ± 24
Rat spleen homogenate	3	52 ± 24
" muscle "	3	82 ± 26
" adrenal "	9	199 ± 19
Murphy-Sturm lymphosarcoma homogenate	4	207 ± 54

* Mean increase plus or minus stand. dev. calculated from paired values before and after inj. for each rat.

TABLE II. Effect of Intravenous Injection of Shear's Tumor Polysaccharide on Plasma Fibrinogen Level.

Dose (μg/kg body wt)	Increase in plasma fibrinogen in 24 hr* (mg/100 ml)
.0 (.2 ml saline)	5 ± 19
.6	34 ± 17
6	115 ± 70
70	159 ± 14
700	156 ± 35

* Mean increase plus or minus stand. dev. calculated from paired values before and after inj. for each rat.

Discussion. These results show that there is a substance in tumor tissue that causes elevation of plasma fibrinogen level when injected into animals. This substance is apparently identical with or closely associated with pyrogenic or tumor-necrotizing polysaccharide isolated from cancer tissue by Shear and his coworkers.

Elevation of plasma fibrinogen level is such a common response to injury or disease that it may well be that there is a common underlying mechanism which can be triggered by a variety of insults to the body. Landy and Shear(5) suggested that damaged tissues release endogenous polysaccharides into the circulation and that these cause the complicated physiological changes which follow injury or infection.

The problem is further complicated by the fact that the different responses to injury occur at different time intervals after injury. Thus, leucopenia develops only a few minutes after injecting endotoxin and temperature elevation begins in 30 to 60 minutes(6) whereas the plasma fibrinogen level is unchanged (or even depressed) for several hours after injecting pyrogen(7).

An investigation of the mechanism underlying the elevation of the plasma fibrinogen level after injury or disease is in progress.

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Two Nutritional Variants of Cultured Jensen Sarcoma Cells.* (24805)

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A previous communication reported that asparagine was essential for growth of the Jensen sarcoma *in vitro*(1). The asparagine requirement could be spared by addition of large amounts of aspartic acid (10-20 mM), but glutamic acid and glutamine were ineffective. During asparagine depletion-repletion experiments, some Jensen cells were observed growing in absence of asparagine. The present report describes development of 2 such nutritional variants of the Jensen sarcoma cultured *in vitro*.

Methods. Tissue culture technics have been described(2). Initial inoculum prepared from intramuscular implants of the tumor in Holtzman rats was 200,000 cells/ml (2 ml total vol. in T-15 flasks). The medium was replenished at end of 48 hours incubation and as often as required thereafter. The substrate used was basal medium 5a(3), and growth was determined at specific time intervals by whole cell count.

Results. When freshly excised Jensen cells were placed in an asparagine-deficient medium most cells became rounded and granular, and total cell population decreased rapidly. At end of 6-7 days of incubation, the number of surviving cells was usually less than 10% of initial inoculum. Some of the remaining cells were viable, since replacing the deficient substrate with the complete medium resulted in cellular proliferation.

When asparagine-depletion experiments were extended beyond an 8-day period, the number of surviving cells continued to decrease and very few viable cells remained. In one such experiment, a different cell type was observed in a flask when the experiment had been in progress for approximately 2 weeks. These cells were elongated and only slightly refractile. Upon continued incubation, no growth was apparent for another week, but after that time there was a definite increase in this particular cell type. As this strain (JA-1) continued to grow, the cells became more refractile and assumed a typical fibroblastic or spindle appearance. There was also a definite tendency to clump or form organoid-like colonies. One month later the JA-1 strain was subcultured and rapid growth was observed. In another series of experiments, a second strain (JA-2) was developed, and the changes which occurred are recorded in Fig. 1.

When JA-1 was implanted into the *rectus femoris* of female Holtzman rats, a tumor developed but at a much slower rate than the Jensen sarcoma. The latency period was approximately 3 weeks as compared to a 4-day latency for the Jensen tumor.

At the end of 5 weeks, strain JA-1 was harvested from the animal and subsequently transplanted into 4 additional rats. Nodules could be palpated after 2 weeks but these regressed after approximately 1 month. Two other attempts to transplant this strain from cells cultured *in vitro* failed, but a 4th try was successful. The period of tumor take was approximately 6 days, but once the tumor had become "established" growth rate was as

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