tions may be lost with it into the large intestine. This is suggested by our observation of several "negative" coefficients. Kunitz (*10*) has reported a stoichiometric, undissociable compound formed by combination of trypsin-inhibitor with trypsin. If significant amounts of secreted proteins are lost in this way, the "methionine-rich" complex observed by Bouthilet *et al.* (*11*) in the feces of chicks fed raw meal could be of endogenous origin. If such lost endogenous protein is high in methionine (relative to soybean protein), the degree of methionine deficiency of animals fed raw meal would be more severe than that estimated by consideration of the amino acid composition of soybeans. Such a sustained loss of secretions may be related to the pancreatic hypertrophy and increased concentrations of trypsinogen and chymotrypsinogen in pancreatic tissue reported in animals fed raw soybeans (*12*).

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Tissue Hemolysins

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Among the many reports on tissue hemolysin published during the past fifty years, cis-vaccenic acid (*1, 2*) has been the only nonspecific hemolysin isolated from tissue extracts and clearly identified. However, the conclusion by Laser (*3*) that there seems to be no need for further speculation regarding their nature appears hardly acceptable in view of the evidence for the hemolysins found in incubated tissue. Laser's procedure for the extraction of the unsaturated acid from horse brain did not clearly indicate any incubation period or definite opportunity for enzymatic activity, thus suggesting the presence of free cis-vaccenic acid or an unstable complex easily broken by the chemical procedure.

If attention is focused on pathological states, it seems relatively unimportant whether hemolysins exist free in normal healthy tissue or whether they are potentially present as enzyme-substrate-inhibitor systems, capable of producing active hemolysin when disturbed by tissue injury or disease. This has led many previous workers to examine diseased tissue, when available, or more often normal tissue, minced, extracted, and incubated sufficiently to produce substantial amounts of active hemolysin. Such treatment has frequently demonstrated (*4–8*) (*a*) that the hemolysins do not arise from contaminating microorganisms; (*b*) that heat, prior to incubation, prevented the development of hemolysins in tissue extracts; (*c*) that a few hours of incubation greatly increased the hemolytic activity; and (*d*) that the active lysin could usually be extracted with alcohol or ether.

TABLE 1

HEMOLYTIC ACTIVITY OF TISSUE HEMOLYSIN AND OLEIC ACID

Mg fatty acid*	Time of complete hemolysis (min)	
	Tissue hemolysin	Oleic acid
0.22	1.5	3.0
.18	3.0	4.5
.09	12.5	40.0
0.07	51.0	> 90.0

* Fatty acid dissolved in 8 ml phosphate buffer, pH 7.3, 2 ml 2% suspension sheep erythrocyte added to each tube.

Experiments in this laboratory have shown that if storage or incubation (4°–37° C), hot solvents, and strong alkaline or acid reagents were avoided, fresh liver, kidney, and testes of rabbit, guinea pig, rat, and cattle yielded no more than traces of hemolytic materials. On the other hand, after a few hours' incubation at 37° C, a strongly hemolytic material could be extracted with cold alcohol.

Current experiments have led to the isolation of a very active hemolysin from incubated saline extracts of bovine testicular tissue. This purified material proved to be a highly unsaturated acid not identical with the octadec-11-enoic acid identified by Morton *et al.* (*2*) in horse brain extracts. It is present in no more than traces in normal healthy tissue, but is readily obtained in the following manner.

Bovine testes were ground, extracted with 0.9% saline, and centrifuged. The supernatant material was titrated to pH 4.5 with acetic acid and centrifuged. The precipitate was suspended in saline at pH 6.2, incubated 8–12 hr at room temperature (20° C), and extracted with ethanol. The active hemolysin in the ethanol extracts was separated from inactive lipoid materials by an extensive series of transfers (detailed elsewhere) between organic solvents and aqueous solutions, at acid, neutral, and alkaline pH. A short bath, low temperature, and low pressure distillation finally yielded a light yellow oil with a molecular weight of 310 + 10 and an iodine number of 257 (Hanus).

Table 1 shows the action of the tissue hemolytic acid in comparison with oleic acid. Hemolysis was inhibited by lecithin but not by cholesterol.

When the precipitate obtained at pH 4.5 was heated at 100° C prior to incubation, no hemolytic

material could be extracted by the process used. When the isolation procedure was carried out rapidly at −2° C, no active hemolysin was found. These ethanol extracts were evaporated under reduced pressure, taken up in ether and precipitated in acetone. This inactive acetone soluble fraction was suspended in phosphate buffer at pH 7.0 and incubated for 2 hr at 37° C with an inactive saline extract of acetone-dried tissue. The incubation mixture of the two inactive fractions yielded an active hemolysin that could be extracted in the manner described above.

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Inactivation of Vaccine Virus by Preparations of Hyaluronic Acid with or without Hyaluronidase: Experiments on Cell Cultures[1]

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The purpose of this preliminary note is to report on the effects of preparations rich in hyaluronic acid, with or without the addition of hyaluronidase, on vaccine virus grown in media of the Maitland type. The materials were added to the cell-containing media, together with the virus, in an attempt to imitate the ground substance of the mesenchyme and thus duplicate, more or less remotely, the conditions that one can imagine prevail during infection (*1*).

We worked with 14 different preparations of either hyaluronic acid or potassium hyaluronate, often highly purified, made from either human umbilical cord or cattle vitreous humor.[3] Saline solutions of these materials were employed (*a*) fresh, after filtration through Berkefeld N candles, (*b*) steamed for 15 min, and (*c*) autoclaved at 120° for 15 min; and at each passage they were added to the cultures, containing 10 ml of medium, generally at the amount of 1 ml of a 1% solution, so that a final concentration of 1:1100 resulted. All the preparations, whether or not they had an inactivating effect on the virus, had a pH ranging from 4.0 to 5.0. Upon addition to the medium the pH of the latter dropped to around 6.8, with some preparations, to return to its normal of 7.2–7.5 shortly after, and remaining so until the end of the culture growth 5 days later. The hyaluronidase preparation[4] was used at a 1% dilution in saline after filtration through Berkefeld N candles. About 200 different lines of cultures, mostly with the Levaditi and dermal strains,[5] were obtained, each line consisting of 1–30 passages. The cultures were titrated on rabbits by intradermal inoculation of progressive tenfold dilutions of the supernatant fluid.

The significant results obtained ranged from a slight decrease in the virus titer to its complete inactivation. This variation on the effects of the polysaccharide preparation depended on the following factors:

a) Source of the preparation: preparations from cattle vitreous humor had less inactivating power than those from human umbilical cord.

b) Concentration of the solution: the inactivating effect was in direct relation to the strength of the solution.

c) Simultaneous addition of hyaluronidase: all preparations that failed to inactivate the virus did so upon addition of hyaluronidase.

d) The culture material employed, whether supernatant fluid or cells, in carrying the passage: the cells afforded extreme protection to the virus.

e) Strain of virus used: dermo vaccine was far more resistant than the Levaditi neuro vaccine.

The order of the neutralizing effect was as follows: Vitreous humor preparations at a final concentration of 1:1100, whether fresh, steamed, or autoclaved, had no effect on the dermo virus other than to reduce the average titer by approximately a one-tenth dilution below the control, whereas the 3 preparations inactivated the neuro vaccine in 3–6 passages. All preparations of umbilical cord at a final concentration of 1:1100 inactivated both strains of virus in 1–5 passages.

Steamed hyaluronic preparations from vitreous humor used at a final concentration of 1:600 completely inactivated the neuro virus, usually in one passage and caused a reduction of two-tenths dilution in the titer of the dermo virus. On the other hand, preparations from both vitreous humor and umbilical cord at final concentrations of 1:10,000 and 1:100,000 did not affect virus growth, or affected it very little.

When the hyaluronic acid preparations inactive at a concentration of 1:1200 were added to the cultures together with the hyaluronidase preparation, also at the final concentration of 1:1200, the virus was totally inactivated after 1–3 passages. The phenomenon is all the more remarkable if one considers that free hyalu-

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[2] With the collaboration of Phyllis Johnson.

[3] Seven of the preparations, supplied by the Wyeth Institute of Applied Biochemistry of Philadelphia, were prepared by H. E. Alburn and E. C. Williams (*2*); another five were prepared and supplied by Z. Hadidian (*3*) from the Tufts Medical School of Boston, and the other two were obtained in our laboratory by R. H. Pearce.

[4] Supplied by J. Seifter, of the Wyeth Institute of Applied Biochemistry. The enzyme contained 1,000 TRU/mg.

[5] The dermo virus, secured through F. L. Horsfall, was the strain cultured by Rivers and Ward (*4*) in 1933 and kept lyophilized since.