including the 300th, produced marked nervous reactions within 24 hours after intracerebral injection. This was true when dilution was carried as high as 1 to 1000. Hamsters exposed by intranasal instillation of 20% brain suspension in amounts as small as 0.06 cc became infected. In exposure by intranasal instillation the incubation period was regularly increased and ranged from 2 to 6 days. Brain suspension from hamsters infected intranasally in the 4th passage titred $10^{-4.77}$ by intracerebral injection, whereas the same form of material from the 7th passage titred $10^{-3.66}$ by the same route. These titrations were made with 0.03 cc of the various brain suspension dilutions. Spinal cord of hamsters of the 300th intracerebral passage, injected in 10% suspension intracerebrally, produced typical nervous symptoms. This suspension of virus titred $10^{-2.5}$, as shown in Table II.

Summary. Infected hamster brains from the 300th intracerebral passage and from the 4th intranasal passage titred $10^{-4.77}$, whereas infected brains from the 7th intranasal passage titred $10^{-3.66}$. Positive Newcastle chicken serum neutralized the virus from the 245th and 300th hamster passage, whereas normal chicken serum had no effect.

Nasal instillation trials were conducted from the 16th and 300th hamster passage but were not successful (except for the 1st passage) until after the 245th passage.

The incubation period of hamsters infected by intranasal instillation is longer than by intracerebral injection. Hamster-adapted Newcastle virus was carried through 9 passages intranasally, and brain material from this 9th nasal passage was neutralized by positive Newcastle chicken serum. Hamsters infected intranasally and intracerebrally showed symptoms of irritability followed by involuntary motor reactions and paralysis.

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17169. The Stability and the Stabilization of Testicular Hyaluronidase.

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Over a period of years, numerous preparations of hyaluronidase have been stored in these Laboratories as bulk powder at room temperature without any appreciable loss in activity. It has been observed, however, that decreases in potency occur when the enzyme is stored under other conditions. We have therefore conducted an investigation of the factors which influence the stability of testicular hyaluronidase. This involved the development of an accelerated aging test and the use of stabilizing agents. The stability of hyaluronidase in bulk, in aqueous solution and in sterile vials has been studied at 10°C, at room temperature and under conditions of accelerated aging. The influence of various bacteriostatic agents on the lability of the enzyme is reported.

Methods. The turbidimetric method of assay employed in this investigation has recently been described in detail.[1] This procedure permits the use of hyaluronates of varying degrees of purity as substrates and gives consistent results because of the stabilization of the protein indicator and the prompt termination of the reaction by heat. The activity of the enzyme is expressed in turbidity reducing units (T.R.U.) which are defined as that amount of enzyme which will hydrolyze hyaluronic acid until it gives a turbidity corresponding to half the amount of substrate employed. Using this method of assay the units have numerical values lower than those obtained by other procedures: *e.g.* 100 TRU

1 Tolksdorf, S., McCready, M. H., McCullagh, D. Roy, and Schwenk, E., *J. Lab. Clin. Med.*, 1949, **34**, 74.

TABLE I.
Loss in Activity of Bulk Hyaluronidase.

Potency of sample TRU/mg	10°C		25-30°C		60°C	
	Time in mo.	% loss	Time in mo.	% loss	Time in mo.	% loss
25	12	0	12	0	1	40
150	12	0	12	0	1	20
500	2	0	3	58	1	62

by this method might be as high as 200 TRU by other methods.

The bulk hyaluronidase was prepared from bull testes by ammonium sulfate and lead acetate fractionation[2,3] and dried from the frozen state. Concentrated solutions of hyaluronidase in pyrogen-free distilled water were sterilized by Seitz filtration and tested for stability at various dilutions. Dilute, sterile filtrates of hyaluronidase were dried in vials from the frozen state. Early experiments indicated that hyaluronidase was less stable in vials than in bulk. Human plasma was added to the hyaluronidase and found to be a suitable stabilizer. Its use however was discontinued due to possible contamination with the virus of infectious hepatitis. It has been noted that certain labile substances such as protein hormones could be stabilized by the use of gelatine.[4] Gelatine which had been purified for intravenous use was therefore added to the vials before drying. This material after drying could be reconstituted only with difficulty. A polypeptide derived from gelatine* was then employed.

The shelf life of hyaluronidase both with and without stabilizer was determined upon storage at room temperature and at 10°C. Since some of the preparations proved to be very stable, an accelerated aging test was devised in order to expedite the study of the stabilization of hyaluronidase. The increased rate of inactivation was studied at various elevated temperatures up to 60°C. Although hyaluronidase in aqueous solution is destroyed almost immediately at 60°C,[1] hyaluronidase in dry form is inactivated at this temperature at a rapid but measurable rate. The stability of bulk powder as well as that of the enzyme in vials was therefore determined by incubation at 60°C for various periods of time.

Hyaluronidase in solution and dried in vials was also tested for stability in the presence of such bacteriostatic agents as zephiran (alkyl dimethyl benzyl ammonium chlorides), phenyl mercuric nitrate and tegosept (methyl and propyl esters of para hydroxy benzoic acid).

Results. I. Stability of Bulk Hyaluronidase. The stability of bulk hyaluronidase is illustrated in Table I. Material assaying 25 TRU and 150 TRU per mg respectively was stable for more than one year when stored at 10°C and at room temperature. Material of higher potency (500 TRU per mg) was stable for several months at 10°C. Stored at room temperature, the 500 TRU material lost 58% of its activity in 3 months. It should be mentioned however, that this study was carried out between June and September, 1948, when the average room temperature was close to 30°C. At 60°C the bulk material, regardless of potency, lost considerable activity over a period of one month.

II. *Stability of Aqueous Solutions of Hyaluronidase.* Aqueous solutions of hyaluronidase in 2% concentration possessed remarkable stability at 10°C. As may be seen from Table II, sterile filtrates of an enzyme preparation assaying 150 TRU per mg were stable for more than 3 months when kept in the refrigerator.

The stability of dilute enzyme solutions was studied at 10°C with 2 preparations assaying 150 and 500 TRU per mg respectively. The

[2] Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

[3] Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

[4] Bockmühl, M., Lindner, F., and Schaumann, O., U. S. Patent No. 2,050,558, 1936.

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TABLE II.
Stability of Concentrated Aqueous Solutions of Hyaluronidase at 10°C.

Experimental*	Original assay, TRU/ml	Time at 10°C, mo.	Final assay, TRU/ml
STE8-gen-20	2580	3	2730
" 22	2640	3	2760
STE9-gen-1B	2590	3	2700
" 6	2670	2.25	2900
" 9	3220	2.5	3260

* Enzyme employed assayed 150 TRU/mg. Concentration of sterile filtrates approximately 2%.

TABLE III.
Loss of Activity of a Dilute Aqueous Hyaluronidase Solution with and without Stabilizer.

	Without stabilizer		With polypeptide	
Enzyme STII-24*	Time	Loss, %	Time	Loss, %
10°	7 wks	50	7 wks	0
Room temp.	7 days	50	18 days	50

* Starting material 500 TRU/mg in 0.028% solution (140 TRU/ml).

500 TRU material at a concentration of 0.28 mg per ml lost 50% of its activity in 7 weeks whereas at the same temperature, in the presence of 2 mg of polypeptide per ml, there was no loss in activity over the same period of time (Table III). At room temperature, the half-life of the enzyme was 7 days without stabilizer as compared to a half-life of 18 days in the presence of polypeptide.

III. Stability of Hyaluronidase in Vials. The lability of hyaluronidase dried from the frozen state in vials in the absence of stabilizer was studied using material assaying 150 TRU per mg and 500 TRU per mg. The vials contained either 5 to 10 TRU, 100 TRU or approximately 150 TRU of the enzyme and were stored at 10°C, at room temperature and at 60°C. Regardless of the amount of hyaluronidase in the vials, the enzyme was stable at 10°C for more than 7 months. At room temperature, losses were frequently observed after 4 months while at 60°C, losses were observed within hours.

The addition of gelatine resulted in a very marked increase in the stability of the enzyme. For vials containing 5 TRU of hyaluronidase, the half-life was 2.5 hours at 60°C without gelatine whereas there was only a very slight loss in the potency of the same preparation in 12 days in the presence of 2 mg of this protein. Unfortunately the contents of the vials were not readily soluble in distilled water.

It was found that a polypeptide produced by the partial hydrolysis of gelatine* was quite soluble after being dried from the frozen state. The stabilizing effects of this polypeptide were the same over a range of 1 to 7.5 mg per vial. Therefore, it was decided to use 2 mg per vial regardless of the quantity and purity of the enzyme. In the presence of this stabilizer the hyaluronidase showed no loss of activity at 10°C and at room temperature for more than 6 months. Some studies made at 60°C are demonstrated in Table IV and are typical of our findings. The results given in the table are expressed in terms of half-life of the enzyme. It will be seen that the half-life of 500 TRU material under these conditions was increased 16 fold. The 150 TRU material had an average increase in half-life from 6.9 to 156 days, indicating a 23 fold increase in stability due to the polypeptide. The vials of batch number 8-gen-17 E contained only small quantities of the enzyme but were stabilized to the same extent as were the other batches. More highly purified hyaluronidase (FVIV-38 C) lost all activity during drying in the absence of stabilizer when the charge was 10 micrograms representing 5 TRU. No activity was lost in the presence of polypeptide. Since the enzyme without polypeptide was stable at room temperature in vials for a period of 4 months, it would seem possible that in the presence of polypeptide the stability at room temperature might be increased to a period of years.

TABLE IV.
Half-Life of Hyaluronidase in Vials at 60°C.

Batch No.	Potency	Vials filled to contain	Half-life	
			Without stabilizer, days	With stabilizer, days
FV IV-38 A	500 TRU/mg	150 TRU/vial	3.5	56
8-gen-17 C	150 " "	165 " "	4.6	163
8-gen-17 A	150 " "	110 " "	14	114
8-gen-17 E	150 " "	9 " "	2	190
FV IV-38 C	500 " "	5 " "	*	85

* All activity lost during drying.

TABLE V.
Stability of Hyaluronidase in Vials During Freeze-Drying.

Batch No	Potency	Content of vials before drying, TRU	Content of vials after drying	
			Without stabilizer, TRU	With stabilizer, TRU
FV IV-38 A	500 TRU/mg	150	120	160
8-gen-17 C	150 " "	165	154	163
8-gen-17 A	150 " "	110	98	114
8-gen-17 E	150 " "	9	4.7	11
FV IV-38 C	500 " "	5	no activity	5.7

Observations were made to determine whether or not a decrease in potency occurred while drying the enzyme in vials. The influence of polypeptide on possible losses during drying was also investigated. Five such experiments are summarized in Table V. In each experiment the same amount of enzyme was placed in the vials with stabilizer as in the control vials. It will be noted that in the absence of stabilizer, losses occured which increased as the amount of enzyme decreased. In the presence of 2 mg of polypeptide, these losses were not observed. During the investigation of solutions of hyaluronidase, it had been noted that the polypeptide did not influence the initial assay values. Therefore it is believed that these experiments indicate that the polypeptide exerts a protective influence over the enzyme during drying.

IV. The influence of Bacteriostatic Agents on the Stability of Hyaluronidase. Tegosept, zephiran and phenylmercuric nitrate were employed in maintaining bacteriostasis in preparing various batches of hyaluronidase. These substances were separated from the enzyme by dialysis prior to drying the final product from the frozen state. Satisfactory preparations were obtained using zephiran and phenylmercuric nitrate. Some preparations in which tegosept was employed became inactive.

Summary. 1. Testicular hyaluronidase in bulk assaying 150 TRU per mg is stable at room temperature for more than one year.

2. Hyaluronidase in concentrated aqueous solution is stable for several months at 10°C. Dilute solutions of the enzyme are less stable.

3. Hyaluronidase dried from the frozen state in vials can be stabilized by the addition of polypeptide. Its half-life at 60°C is thereby extended 10 to 20 times.

4. Certain bacteriostatic agents can be used throughout the production of hyaluronidase without decreasing the activity of the enzyme.

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