Effect of Hyaluronidase on Toxicity of Procaine and Tetracaine in Mice.* (19554)

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Hyaluronidase has been added to local anesthetic solutions used in regional anesthesia. This mucolytic enzyme will hydrolyze the hyaluronic acid barrier found in various tissues of the body and thereby promote diffusion of the injected fluid(1-7). Although anesthesia may be more complete under certain situations with the use of hyaluronidase some investigators have either observed or are of the opinion that there is an increased incidence of systemic toxic reactions from the local anesthetic when hyaluronidase is added (3,4,7). Therefore experiments were conducted to determine the effect of hyaluronidase on the systemic toxicity of procaine and tetracaine injected subcutaneously into mice.

Methods and materials. The LD_{50} of procaine and of tetracaine alone and in combination with hyaluronidase were determined by injecting the solutions subcutaneously into white mice of either sex and weighing 20 to 30 g. Fresh solutions of procaine hydrochloride (Merck) and tetracaine hydrochloride (Winthrop-Stearns, Inc.) were prepared in 0.9% sodium chloride solution. Fresh solutions of hyaluronidase† containing either 150 T.R.U. or viscosity units per ml were prepared in 0.9% sodium chloride solution. The hyaluronidase solution was mixed with the local anesthetic solution in the syringe immediately before injection. It was decided to keep the total volume of solution to be injected into each mouse constant irrespective of the weight of the mouse and the dose of the local anesthetic. Therefore each mouse received a total volume of 0.3 ml of solution of which 0.05 ml was either 0.9% sodium chloride solution or the hyaluronidase solution and the remaining 0.25 ml contained the required amount of local anesthetic agent. The final hyaluronidase concentration was 25 units per ml. Additional experiments were conducted to determine the influence of hyaluronidase on the blood level of procaine following its subcutaneous injection in mice. The mice were from the above mentioned animal stock. The preparation of the solutions and the method of injection were the same as that already mentioned. The amount of procaine in the blood was determined with the method of Brodie *et al.*(8) which is a modification of the procedure for determining sulfonamides. The mice were sacrificed by a blow on the head at various time intervals after the subcutaneous injection of 435 mg/kg of procaine hydrochloride alone or mixed with the hyaluronidase. Blood was removed by cardiac puncture and placed in a tube containing sodium oxalate. Within one minute, 0.2 ml of this blood was placed in a 50 ml glass-stoppered flask containing 0.5 ml of a 50% solution of sodium arsenite and 1.0 ml of distilled water. Three minutes later 1.0 ml of 0.8 M borate buffer (pH 9) and 20 ml of ethylene dichloride were added. The other modifications of Brodie *et al.* procedure were that 3.0 ml of 1 N HCl was used in place of 1.0 ml to extract the procaine from the ethylene dichloride and the volume of sodium nitrite and subsequent solutions was increased to 0.15 ml. The optical densities of the solutions were determined at 550 μ in a Model 6A Coleman Junior Spectrophotometer. Preliminary experiments demonstrated that the recovery of procaine when added to blood or plasma in amounts of 1.25 to 3.75 μg was 96 $\pm$ 3%.

Results. Table I summarizes the results of the effect of hyaluronidase on the systemic toxicity of procaine injected subcutaneously in mice. The method of Litchfield and Wilcoxon(9) was used to calculate the LD_{50}. It was found that at the 19/20 confidence limits the systemic toxicity of procaine was

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TABLE I. Summary of Effect of Hyaluronidase on Systemic Toxicity of Procaine Hydrochloride Injected Subcutaneously in Mice. Volume of solution injected –.3 ml; hyaluronidase conc. –25 units/ml.

	Control					With hyaluronidase					
Dose, mg/kg	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	Probability of t value
370						10	0	10	7.1	± 3.3	
400	10	1	8	6.5	± 2.7	10	1	10	4.7	± 1.9	
435	15	0	7	5.2	± 2.4	15	7	13	4.8	± 9.2	
510	15	3	15	5.6	± 2.3	15	7	13	3.9	± 1.3	
550	10	4	9	5.6	± 3.1	10	5	10	4.6	± 2	
600	15	5	15	4.8	± 2.1	15	12	15	2.9	± 1	.2–.5
700	10	5	10	7	± 6.6	10	4	10	3.3	± 1.8	.1–.2
800	10	8	10	2.9	± 1.1	10	9	10	2.3	± .7	
900	10	9	10	3	± 1.6	10	9	10	3	± 1.5	
LD_{50}	655 mg/kg					540 mg/kg					
5% limits	582-737 mg/kg					482-605 mg/kg					
	Potency ratio, 1.22 (significant)										

from 1.04 to 1.42 times greater when combined with hyaluronidase. However, when calculated at the 99/100 limits, the addition of hyaluronidase did not increase significantly the systemic toxicity of procaine. This LD_{50} for subcutaneously injected procaine is lower than that reported by Ting and Coon(10) but within the range reported by other investigators(11).

The time of onset of convulsions was recorded to the nearest one-fourth minute in an attempt to determine whether hyaluronidase increased the rate of absorption of the subcutaneously injected procaine. The appearance of convulsions was considered to be the most convenient sign of onset of systemic toxicity from procaine. Applying the "Student" t test at the 600 and 700 mg/kg dose levels it was observed that the addition of hyaluronidase did not change significantly the average time of onset of convulsions produced by the procaine.

One explanation whereby the combination of hyaluronidase with procaine did not increase its systemic toxicity could be due to a rapid rate of breakdown of procaine in the subcutaneous tissue. This problem was investigated by determining the procaine blood level of mice following the subcutaneous injection of procaine hydrochloride alone and in combination with hyaluronidase. The results are shown graphically in Fig. 1. This dose of procaine was selected with the opinion that the possible role of procaine destruction at the site of injection could be demonstrated more readily at a lower dose level. It was observed that procaine injected without hyaluronidase appeared in the blood of mice within one minute following the injection. The procaine blood level increased rapidly between the second and the eighth minute after the injection. Between the eighth and twentieth minutes, the level remained fairly constant except for the fall at

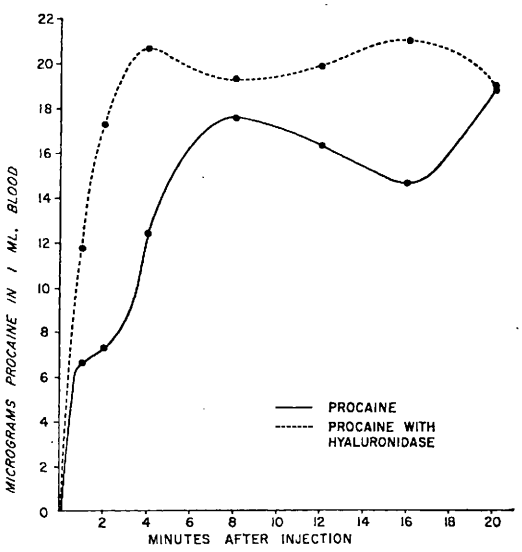

FIG. 1. Mean procaine blood level in mice following subcutaneous injection of 435 mg/kg of procaine hydrochloride alone and with hyaluronidase. Volume of sol. inj. .3 ml, hyaluronidase conc. 25 units/ml. Each point on curves are results from 3 to 5 mice. Probability of t value at: one min .1 to .2, two min .02 to .05, four min >.01, eight min .2 to .5, sixteen min .01 to .02.

TABLE II. Summary of Effect of Hyaluronidase on Systemic Toxicity of Tetracaine Hydrochloride Injected Subcutaneously in Mice. Volume of solution injected, .3 ml; hyaluronidase conc., 25 units/ml.

	Control					With hyaluronidase				
Dose, mg/kg	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.	No. inj.	No. died	No. react.	Avg time onset of reactions, min	St. dev.
20	10	0	7	4.6	± 1.4	10	1	9	3.5	± 1.2
30	10	1	10	3.3	± .9	10	4	10	3.3	± 2.5
40	10	5	10	3.4	± 1.5	10	6	10	2.5	± .6
50	10	6	10	3 *	± 1.1	10	7	10	2.1*	± .6
60	10	7	10	2.4	± .3	10	10	10	1.8	± .5
70	10	9	10	2.1	± .3					
LD_{50}	44 mg/kg					36 mg/kg				
5% limits	35-55 mg/kg					28-45 mg/kg				
	Potency ratio (not significant)									

* Probability of t value = >.5.

the 16-minute interval. Procaine injected with hyaluronidase resulted in a more rapid increase of the procaine blood levels. Using the "Student" t test, the procaine blood levels were significantly higher than the controls at the 2 and 4-minute periods. However, with the exception of the 16-minute period, there was no significant difference in the maximal blood procaine level attained in the 2 groups of animals.

It was noted during the experiments that there was no correlation between a given procaine blood level and the occurrence of convulsions.

After observing that the combination of hyaluronidase with procaine, increased significantly the systemic toxicity of procaine at the 5% probability, similar experiments were conducted to determine the effect of hyaluronidase on the systemic toxicity of tetracaine. This local anesthetic was selected because of its greater toxicity as compared to procaine. The statistical methods were the same as those used in the previous experiments. The results are summarized in Table II.

It was observed that the combination of hyaluronidase with tetracaine injected subcutaneously did not increase significantly the LD_{50} of tetracaine. Furthermore the addition of hyaluronidase did not significantly shorten the time for onset of convulsions.

The LD_{50} for tetracaine was the same as that reported by Fussganger *et al.*(12).

Summary. It was observed that only at the 19/20 probability limits did hyaluronidase increase the toxicity of procaine injected subcutaneously into mice. Hyaluronidase did not increase the toxicity of tetracaine within these same limits. From determinations of the procaine in blood of mice at various time intervals after its subcutaneous injection, it was observed that the procaine blood level increased rapidly beginning with the second minute and reached its peak at the eighth minute. The procaine blood level remained uniform between the eighth and twentieth minutes after injection. When hyaluronidase was mixed with the procaine injected subcutaneously there was a more rapid rate of increase of the procaine blood level than that of the control but the maximal procaine blood levels in the 2 groups were the same.

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Antifatty Liver Action of Papain and Ficin in Insulin-Treated, Depancreatized Dogs.* (19555)

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Previous work from this laboratory has shown that the fatty liver that develops in insulin-treated, depancreatized dogs can be prevented by the feeding of either methionine, choline. or hydrolysed casein(1,2). On the basis of these findings, it was proposed that the prevention of the fatty liver by raw pancreas, pancreatic juice and certain fractions derived from raw pancreas was the result of proteolytic enzymes furnished by the pancreatic substances(3). This view was later substantiated by the finding that crystalline trypsin, when added to the diet, also prevented the development of fatty livers(4).

The present report shows that prevention of fatty livers in depancreatized dogs can be achieved with plant preparations—papain and ficin—substances rich in proteolytic activity.

Experimental. The methods for assay of antifatty liver activity have been described elsewhere(5). Papain and ficin were fed twice daily to depancreatized dogs, for periods of 18-21 weeks, along with a diet consisting of 250 g of lean meat, 50 g of sucrose, 5 g of bone ash, and 1.5 g of salt mixture(4). Vitamin supplements(4) were fed once daily. Eight units of insulin were injected at each time of feeding. During the 3-4-week interval between pancreatectomy and the initiation of papain and ficin administration, the dogs were fed 25 g of raw pancreas with each meal in order to insure normal livers at the start of the assay period. At the end of the assay period, the dogs were anesthetized with nembutal. The entire liver was removed and thoroughly ground and mixed. Several samples were taken for the determination of total fatty acids(6).

Results. The fatty acid content of the livers of the control dogs fed no supplement ranged from 13.2 to 21.5% (Table I). The livers of the dogs fed papain were well within the normal range, *i.e.*, from 2.2 to 4.7%. Similar results were obtained with ficin-fed dogs; in these, the total fatty acid content of the liver varied from 1.6 to 2.5%. Throughout the course of the experiment the animals displayed a vigorous appetite. Ficin and papain did not prevent a loss in weight in these dogs.

Discussion. The capacity of papain to digest protein is well established(7), and its specificity, as determined on certain peptide linkages, is apparently similar to that of trypsin; indeed, papain is classified as a trypsinase (8). It is of interest to note that papain shows proteolytic activity over a wide range of temperature (10°-70°) and pH (3-12)(7). Although ficin has not been so extensively studied, it is nevertheless considered a papain-like enzyme(9).

Procedures for preventing the accumulation of fat in the liver of insulin-treated, depancreatized dogs can be classified into 1) those furnishing lipotropic substances in the free

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