Stability of Pepsin During Storage in Canine Gastric Secretions and in Aqueous Solutions* (33764)

MARIAN C. HUNNER, FRED B. HUDSON, AND T. LLOYD FLETCHER

Chemistry Research Laboratory of the Department of Surgery, University of Washington School of Medicine, Seattle, Washington 98105

Since we are required to assay the pepsin content of a large number of samples of gastric secretion from experimental pouch dogs, we have studied the stability of pepsin, at the low concentrations consistent with our assay method, at various temperatures, seeking a satisfactory method of storage and attempting to clarify some confusion in the literature.

Materials and Methods. Canine gastric secretions (CGS) and commercial crystalline porcine pepsin (CCPP) solutions (5 and 10 μg/ml) were assayed following storage for up to 20 days at 23–28, 4, 0 (ice bath), — 4 and — 20°.

We also studied the effects of added bovine and canine albumin on these solutions, stored at —20° with and without acidified glycerol (1, 2).

Gastric secretion was collected by gravity flow through a cannula inserted in Heidenhain (denervated) or Pavlov (innervated) pouches or from a gastric fistula (5 dogs). From 3 to 12 samples (0.5 hr), collected from each dog, were chosen at random and were filtered and adjusted to pH 1.8 with 0.024 *M* HCl, and assayed. This is the optimum pH for pepsin hydrolysis of hemoglobin. In our laboratory, the coefficient of variation for this method is 5%.

Crystalline porcine pepsin was obtained from two manufacturers;[1] the two were used interchangeably.

Enzyme activity was determined by Anson's (3) modification of a method of Anson and Mirsky (4). Measurement of pepsin activity (1-ml samples) was in Anson-Mirsky units (5). One such unit represents the release of 5.52 μmoles of tyrosine from 2.5% denatured dried hemoglobin substrate at pH 1.8, during 15 min incubation at 37° (or 0.370 μmoles of tyrosine/min which is 370 IU of pepsin/liter). Optical density was read at 280 mμ.

The stock solution contained 0.50 mg of crystalline pepsin/ml of 0.024 *M* HCl. Two working standard solutions were prepared from this (5 and 10 μg/ml) in 0.024 *M* HCl (pH 1.8).

Acidified glycerol (pH 1.8) was prepared by diluting 95% glycerol (C.P.) 1:1 with 0.0246 *M* HCl. This was added to the CGS or the CCPP solutions, which were stored as 1-ml aliquots.

Acidified solutions (pH 1.8) of canine albumin, Fraction V, [1a] (100 mg/100 ml) and of bovine albumin, Fraction V,[1a] (50 mg/100 ml, 100 mg/100 ml and 200 mg/100 ml) were prepared by dissolving albumin in 0.024 *M* HCl.

Results. The 110 CGS samples showed fluctuations in pepsin activity following storage at 23–28° (room temperature) for 5 hr, or at 4° (refrigerator temperature) for 3 days, or at —4° (freezing compartment) for 2 days; whereas at 0° (ice bath) early fluctuations were followed after 3 hr by some stabilization. The CCPP solutions, on the other hand, showed little loss in activity until after 5 hr at 0°, but a steadily increasing loss occurred on longer storage (1–13 days) with greater increases at lower temperatures (Table I).

At —20° CGS samples showed a rapidly increasing loss in activity; at 8 days the loss was 93%. The CCPP samples showed an 83% loss at 13 days (Fig. 1). Glycerinized CGS samples, stored 1 day at —20°, lost 3% of

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[1] (a) Nutritional Biochemicals Corporation, Cleveland, Ohio 44128; and (b) Worthington Biochemical Corporation, Freehold, New Jersey 07728.

TABLE I. Changes in Pepsin Activity[a] in Canine Gastric Secretions (CGS)[b] and in Commercial Crystalline Porcine Pepsin (CCPP)[b] without Added Glycerol or Albumin.

	Storage temp															
	23–28°				4°				0°				−4°			
	CGS		CCPP		CGS		CCPP		CGS		CCPP		CGS		CCPP	
Time	Mean[c]	Range[d]	Mean[c]	Range[d]	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
(hr)																
0.5	98[e]	80–108	—	—					92[e]	75–104	—	—				
1.0	98	84–110	97[f]	88–100					101	98–105	97[f]	96– 98				
2.0	87	85–111	99	88–108					85	75–108	110	108–112				
3.0	97	87–105	93	88–103					98	96–100	99	96–103				
4.0	94	98–100	92	82–105					95	92– 97	97	94–100				
5.0	96	94–100	92	82–101					92	91– 96	100	100–101				
(days)																
1					91[g]	62–158	87[h]	84–90					116[e]	91–155	60[i]	54–65
2					86[j]	72–100	90	81–98					86[f]	73–100	52	51–52
3					111[k]	82–141	90	83–96					—	—	52	47–57
4					—	—	92	88–96					—	—	52	44–60
5					—	—	—	—					—	—	—	—
6					—	—	—	—					—	—	—	—
7					—	—	—	—					—	—	—	—
8					—	—	88	86–90					—	—	42	35–48
9					—	—	78	72–85					—	—	—	—

[a] Standard deviation is ± 5%.

[b] All solutions pH 1.8.

[c] Mean of percentage pepsin activity remaining; CCPP column includes results for both concentrations of the enzyme.

[d] Range of percentage pepsin activity remaining; CCPP column includes results for both concentrations of the enzyme.

[e] Five expts. [f] Ten expts. [g] One hundred ten expts. [h] Four expts. [i] Two expts. [j] Twenty-two expts. [k] Twenty-eight expts.

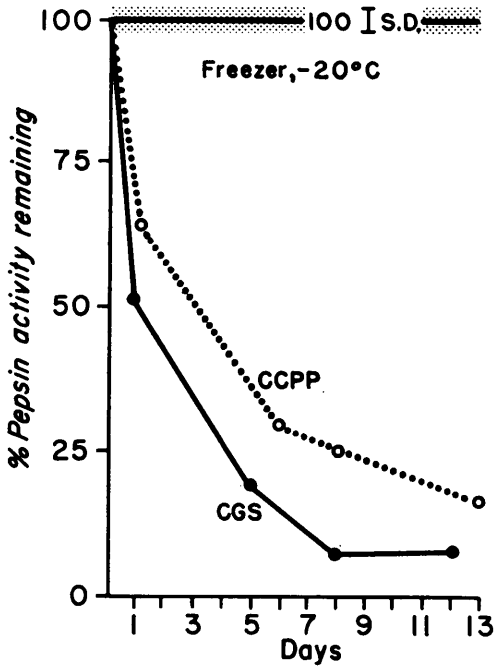

FIG. 1. Effect of frozen storage on pepsin activity in canine gastric secretion (CGS) and commercial crystalline porcine pepsin (CCPP) without added glycerol or albumin: all solutions (pH 1.8) were thawed in cold running water; CCPP values are the mean of all results (8 experiments) for both concentrations; standard deviation, ± 5%.

their activity; results after further storage are shown in Fig. 2.

Addition of both glycerol and canine albumin (100 mg/100 ml) stabilized CCPP solutions stored at —20° for up to 6 days (Fig. 3). Figure 4 shows the effect of different concentrations of added albumin, with and without glycerol, on the pepsin activity of CCPP solutions stored at —20° for as long as 20 days. These low concentrations of CCPP showed best retention of pepsin activity with both 100 mg/100 ml of added albumin, the approximate concentration of total protein (biuret method) found in CGS, and with added glycerol. In these solutions there was 97% retention of pepsin activity at 6 days.

These are the best methods we have found for stabilization of pepsin activity in CGS and CCPP solution. The CGS can be held in an ice bath at 0° for up to 3 hr or can be stored for 1 day at —20°, if combined with acidified glycerol. The CCPP solutions of these concentrations can be held up to 5 hr at 0°. For longer storage (up to 6 days) samples must be combined with albumin or, better, with albumin and acidified glycerol.

Discussion. Acidification of pepsinogen, found in gastric mucosa, yields pepsin. In 1936, Herriott (6) reported only "minor losses of peptic activity" of pepsin in acid solution when stored at 5° "for weeks." Two years later Ihre (7) stored human gastric juice in a refrigerator at pH 0.95 for 22 days and at pH 1.98 for 75 days "with little change in proteolytic activity." However, it was 1964 before it was pointed out (8) that the pH for maximum stability of pepsin varies with species.

In 1938, Herriott (9) showed that the pepsin formed from pepsinogen resulted partly from an autocatalytic reaction, the presence of pepsin accelerating its own formation. Recently, Rajogopalan *et al.* (10) presented evidence of the heterogeneity of CCPP.

Fluctuation in pepsin activity of our CGS samples occurred at all temperatures (Table I); at —20° a precipitous loss occurred. At 8 days the loss was 93% (Fig. 1). Similar variations in concentration were found by Baker *et al.* in their study of plasma protein components stored at 22, 4, and —20° for 16 days. (11) They suggested that instability as a result of storage might be due to con-

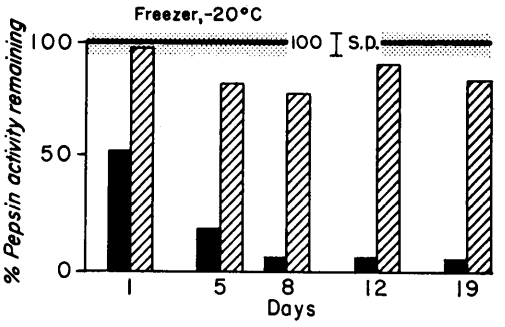

FIG. 2. The effect on pepsin activity, in frozen storage, of addition of acidified glycerol (1:1 with 0.024 *M* HCl) to CGS, (square with diagonal lines); compared with CGS solutions without glycerol, ■; all solutions were pH 1.8; frozen samples were thawed under cold running water; standard deviation, ± 5%.

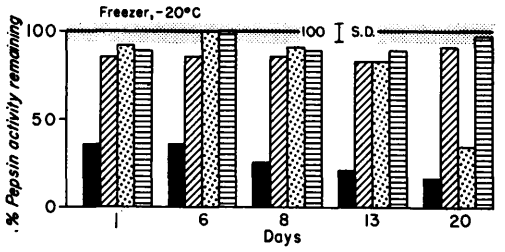

FIG. 3. Comparison of the effect on pepsin activity of CCPP solutions stored (frozen), as follows: CCPP, alone, ■; CCPP with acidified glycerol (1:1 with 0.024 *M* HCl) (square with diagonal lines); CCPP with canine albumin, 100 mg/100 ml (square with dots); and CCPP with canine albumin, 100 mg/100 ml, in acidified glycerol (square with horizontal lines); all solutions were pH 1.8; values are the mean of results for both concentrations (5 and 10 μg/ml); standard deviation, ± 5%.

formation changes. Another possible clue was pointed out by Grant and Alburn (12) who found acceleration of certain enzyme reactions in the frozen state.

Variation in the amount of pepsin activity is not due to the presence of pepsin-inhibitor complex as postulated at first by Bucher *et al.* (13). Bucher (14) and Muther (15) later found that the apparent inhibition was caused by substrate exhaustion with undiluted gastric juice of high pepsin concentration. We avoided this by selecting a volume adjusted to the content of pepsin which was in the linear range of the Anson and Mirsky method.

The CGS samples with acidified glycerol retained pepsin activity at —20° for 1 day (Fig. 2); this was not true for CCPP, however (Fig. 3). Grossman and Marks (16) stored a more concentrated pepsin standard, 1 mg/ml, in equal volumes of 0.01 *M* HCl and glycerol at —20° for as long as 6 months "with no deterioration in activity." With these more dilute pepsin solutions we found, however, that addition of 100 mg/100 ml of albumin, or this concentration of albumin combined with acidified glycerol, to both concentration of CCPP was the best method for retention of pepsin activity for storage more than one day at —20° (Fig. 3). This approach was prompted particularly by the rapid loss in activity of the dilute CCPP solutions, together with the realization that its protein content was much lower than that of the CGS solutions. In general, CCPP solutions did not show the erratic fluctuation in pepsin activity which occurred with CGS solutions, but showed steadily increasing losses both with time and as temperatures were lowered.

Summary. We have determined the pepsin activity of canine gastric secretions (CGS) and of commercial crystalline porcine pepsin (CCPP) solutions at pH 1.8 (5 and 10 μg/ml) for various periods of time, at room temperature (23–28°), at refrigerator temperature (4°), in the freezing compartment of a refrigerator (—4°) and at —20°. Lowered activity was found under most of these conditions. The lower the temperature and the longer the time the greater is the loss. The CGS samples deviated somewhat from this general trend by showing some increases as well as decreases in activity at all temperatures except at 0°. We, therefore, recommend holding such samples in an ice bath at 0° for not longer than 3 hr, or addition of acidified glycerol for storage up to 1 day at —20°. Fresh preparations of CCPP solutions retained activity in an ice bath for up to 5 hr. If it is necessary to store CCPP solutions for a longer period of time—up to 6 days—addition of albumin or a combination of albumin and acidified glycerol is essential.

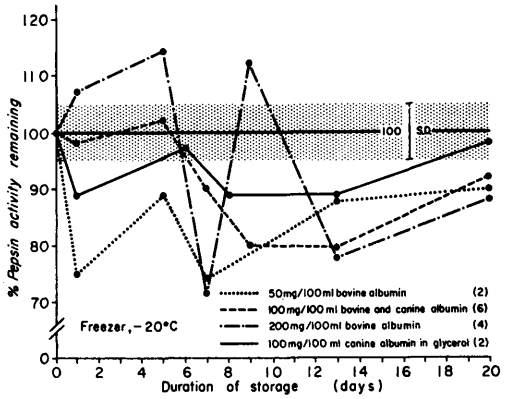

FIG. 4. Effect on pepsin activity of addition of different concentrations of bovine or canine albumin to commercial crystalline porcine pepsin (CCPP) solutions (pH 1.8) during frozen storage; numbers in ( ) are the number of experiments; standard deviation, ±5%.

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The Action of Phenacetin on Tremorine-Induced Cholinergic Effects (33765)

D. A. McCLURE (Introduced by T. Ellison)

Department of Biological Sciences, Riker Laboratories, Northridge, California 91324

In laboratory animals, tremorine is a potent analgesic agent. In addition, it possesses profound cholinergic activities which include the peripheral effects of diarrhea, chromodacryorrhea, salivation, and centrally-induced tremors. It has been shown by Kocsis and Welch (1) and Cho *et al.* (2) that tremorine is metabolized into oxotremorine which is many times more potent than tremorine. This conversion, which occurs in the liver, was shown to be blocked by the liver microsomal enzyme inhibitor SKF 525-A (3). In addition, phenothiazines act similarly to SKF 525-A in that they block the tremors in mice induced by tremorine but not those induced by oxotremorine (4). Anticholinergic compounds block the effects of both tremorine and oxotremorine and probably do not enter into the biological conversion mechanism.

During the course of investigating potentially active analgesic agents, various amounts of APC (aspirin, phenacetin, and caffeine) were used in conjunction with the agents in an attempt to augment their analgesic activity. It was shown previously (5) that the addition of APC to analgesic agents enhances their activity by an unknown mechanism.

Preliminary experiments in our laboratory showed that the administration of APC compound not only augmented the analgesic effect of tremorine but also markedly reduced the cholinergic effects. Further investigation revealed that neither aspirin nor caffeine altered the tremorine-induced cholinergic effects, whereas phenacetin tended to delay the onset of tremorine action. This report concerns the interaction of phenacetin with tremorine and oxotremorine particularly with respect to the cholinergic effect of the latter two compounds.

Material and Method. The tremorine effects of diarrhea, chromodacryorrhea, salivation and tremors were quantitatively measured in 100 mice. A dose of 5 mg/kg i.p.