HEMATIN FORMATION AND PANCREATITIS

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HEMORRHAGIC PANCREATITIS is a more lethal disease than edematous pancreatitis [2, 5, 8]. For prognostic and therapeutic purposes it would be useful to be able to distinguish between the two forms of pancreatitis, but present methods are inadequate. Levels of serum amylase, an index of the degree of ductal obstruction of the pancreas, depend on a functioning acinar cell [1]. Amylase and lipase levels do not reflect the presence of parenchymal necrosis unless diffuse destruction alters the capacity of the pancreas to produce amylase and lipase through a marked reduction in the number of viable acinar cells. Desoxyribonuclease I has been used as a measure of acinar cell destruction, but the method is expensive and time-consuming.

Northam [7] has developed a test for detecting hemorrhagic necrosis of the pancreas based on the appearance of hematin in the serum, which is attributed to the action of pancreatic enzymes on hemoglobin released from blood extravasated into the peritoneal cavity and interstitium of the pancreas. Presumably the hematin formed within the pancreas and peritoneal cavity reaches the bloodstream by way of the venous capillaries and abdominal lymphatics.

Hematin may appear in the serum as a result of diseases other than pancreatitis [9]. Any disease causing intravascular hemolysis results in hemoglobin release into the serum. The released hemoglobin is complexed with haptoglobin and rapidly excreted. Hemoglobin continues to be bound to haptoglobin as long as serum haptoglobin is available. During conditions of intravascular hemolysis free hemoglobin and its breakdown product hematin will appear in the serum only if the rate of hemolysis exceeds the capacity of haptoglobin to bind hemoglobin. Under such circumstances serum analysis would show free hemoglobin and hematin, and saturation of all haptoglobin by hemoglobin [7]. Northam felt that intraperitoneal and not intravascular hemolysis was the source of serum hematin in patients with hemorrhagic pancreatitis, because unbound haptoglobin was also present in their serum.

Other sources of intraabdominal hemorrhage from, for example, trauma or ectopic pregnancy are not likely to be associated with the appearance of serum hematin [7]. Northam asserted that most blood released

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into the peritoneal cavity is not quickly degraded to hematin in the absence of pancreatic enzymes. However, he offered no experimental evidence to substantiate his belief that pancreatic enzymes could accelerate red cell breakdown and conversion of hemoglobin to hematin.

The purpose of this study is to evaluate the capability of specific pancreatic enzymes to break down red cells and convert ferrous hemoglobin to ferric hematin.

MATERIALS AND METHODS

Formation of a Standard Hemoglobin Solution

Human red cells were centrifuged at 13,000 rpm after removal of the serum. The red cells were washed 4 times with sterile isotonic saline and lysed by standing for 20 minutes at room temperature with an equal volume of sterile water. This solution was centrifuged for 15 minutes to remove cell ghosts. The supernatant was separated and became the stock solution of hemoglobin.

The hemoglobin concentration was determined by the Shinowara "oxygen capacity method" after dilution of the stock solution [11]. The appropriate amount of diluted stock hemoglobin was then added to the tubes to make the solutions to be incubated approximately 100 mg. per 100 cc. of hemoglobin.

Experiment IA

The effect of a mixture of pancreatic enzymes on the conversion of hemoglobin to hematin was compared with that of fresh pancreatic juice.

The enzymes employed were lecithinase (.0075 gm.), elastase (.0030 gm.), lipase (.0051 gm.), collagenase (.0021 gm.), enterokinase (.0024 gm.), trypsin (.0033 gm.), and amylase (.0025 gm.).* These enzymes were dissolved in 10 ml. of saline immediately prior to use.†

Three tubes were prepared containing 0.1 ml. of the diluted stock hemoglobin solution (10 gm.%), 2 ml. of M/10 phosphate buffer (pH 7.4), 0.1 ml. of saline, and water sufficient to make 10 ml. after the enzymes were added. To one tube 0.5 ml. of the mixture of pancreatic enzymes was added, to the second tube 0.5 ml. of freshly thawed human pancreatic juice (frozen at −40°C.), and to the third tube, which served as a blank, water alone.

The solutions in each tube were mixed and read on the Beckman D.U. spectrophotometer as close to zero time as possible. The 3 tubes were then incubated in a moving water bath at 37°C. Readings were obtained at 23, 53, 124, 181, and 282 minutes at wave lengths of 675, 615, 575, 560, and 450.

The concentrations of bilirubin (C_b), hemoglobin (C_h), and hematin (C_t) were calculated by the Shinowara method [11].‡

$$C_b = 1.27A_{450} - 1.35A_{575} - 2.19(A_{615} - A_{675})$$

$$C_h = 1.31C_b + 256(A_{575} - A_{560}) + 38.4(A_{615} - A_{675} - .02)$$

$$C_t = -0.08C_h + \frac{10^3}{3.02}(A_{615} - A_{675} - .02)$$

where

A_x = optical density at x wave length, and C_b, C_h, and C_t are given in mg.%.

Experiment IB

The effect of time on loss of potency of the pancreatic enzymes was evaluated. The procedure used in Experiment IA was repeated with the same pancreatic enzymes in Experiment IB. The only difference was that the pancreatic enzymes used in Experiment IB

*Nutritional Biochemicals, Cleveland, Ohio (lecithinase D and enterokinase); Mann Research Laboratories, subsidiary of B&D Laboratories, New York, N.Y. (elastase); Steapsin Hog Pancreas Chemical Co., St. Louis, Mo. (lipase); Worthington Biochemical Co., Freehold, N.J. (collagenase and amylase); Armour Pharmaceutical Co., Kankakee, Ill. (tryptor-trypsin).

†The weights of enzymes used represent amounts that experimentally gave a saturated solution in 10 ml. of saline when mixed at room temperature.

‡To calculate the bilirubin concentrations the serum absorbency factor was eliminated.

were 24 hours old rather than mixed fresh as in Experiment IA.

Experiment II

The pancreatic enzymes employed as a mixture in Experiment I were studied separately to determine their effect on the conversion of hemoglobin to hematin with and without enterokinase. The same weight of enzymes dissolved in 10 ml. of saline as in Experiment I was employed. A reagent solution was prepared containing 1 ml. of diluted hemoglobin stock solution, 20 ml. of M/10 phosphate buffer (pH 7.4), 1 ml. of normal saline, and enough water to give a volume of 84 ml.

To each of 2 tubes to be incubated was added 4.2 ml. of the reagent hemoglobin solution. Enterokinase (0.3 ml.) was added to one tube and water to the other. Pancreatic enzymes (0.5 ml.) were then added to each tube. The enzymes employed in individual experiments were lecithinase (.0075 gm.), elastase (.0030 gm.), lipase (.0051 gm.), collagenase (.0021 gm.), trypsin (.0035 gm.), and amylase (.0025 gm.). In addition a blank was prepared containing 4.2 ml. of hemoglobin reagent solution plus 0.8 ml. of water. The solutions in each tube were mixed and read on the Beckman D.U. spectrophotometer at time periods of 15 to 67, 178 to 239, 524 to 564, and 1270 to 1322 minutes, and the concentrations of bilirubin, hemoglobin, and hematin were calculated as in Experiment I.

Experiment IIIA

In both parts of Experiment III the effect of pancreatic enzymes on hematin formation from red cells rather than from hemoglobin was studied as in the earlier experiments.

One ml. of whole blood was suspended in 50 ml. of saline. The blank tube consisted of 4.5 ml. of the saline-blood suspension plus 0.5 ml. of saline. A second tube contained 4.5 ml. of the saline-blood suspension plus 0.5 ml. of a saturated solution of amylase, lecithinase,, and collagenase. In Experiment II these enzymes had shown the least activity in converting hemoglobin to hematin. The third tube was similar to the second, but contained 3 different enzymes: elastase, trypsin, and lipase. These enzymes had been the most rapid producers of hematin in Experiment II. The fourth tube was similar to the second and third tubes except that it contained all 6 pancreatic enzymes plus enterokinase in saturated solution. The 4 tubes set up were read on the Beckman D.U. spectrophotometer at time zero. Readings were accomplished by gently spinning down the cell suspension at 2,000 rpm for 2 minutes and decanting enough supernatant for reading. After reading, the sample was returned to the solution tube and the tubes were then gently inverted. The Beckman D.U. spectrophotometer was set at zero with supernatant from the blank. Concentrations of hematin, bilirubin, and hemoglobin were determined as in Experiments I and II. Readings were obtained at 15, 88, 181, 294, 409, and 532 minutes.

Experiment IIIB

The enzymes which showed the most activity in breaking down red cells and converting hemoglobin to hematin were individually tested.

The same concentrations of lipase, elastase, and trypsin used in Experiment IIIA were added individually to the red blood cell suspensions. Readings on the Beckman D.U. spectrophotometer were obtained at 70, 165, 300, and 375 minutes.

RESULTS

Experiment IA

Both mixed pancreatic enzymes and freshly thawed human pancreatic juice were capable of accelerating the conversion of hemoglobin to hematin in comparison to the normal decay of a phosphate-buffered hemoglobin solution. The mixture of powdered enzymes proved to be faster in action than the human pancreatic juice but similar in its effect on hemoglobin. Hemoglobin levels fell and bilirubin levels increased during the conversion of hemoglobin to hematin (Table 1). Grossly the tubes with pancreatic enzymes turned brown with precipitate while the blank solution remained red.

Table 1. Activity of Mixture of Powdered Pancreatic Enzymes and Freshly Thawed Human Pancreatic Juice Measured by Production of Hematin from Hemoglobin

Agent	Time (min.)	C_b[a] (mg. %)	C_h[a] (mg. %)	C_t[a] (mg. %)
Blank	23	.067	99.34	−9.27
	53	.045	97.26	−7.45
	124	.041	93.73	−4.52
	181	.030	91.99	−4.05
	282	.024	86.30	−1.94
Mixture of pancreatic enzymes[b]	23	.085	96.70	−7.40
	53	.061	92.96	−6.14
	124	.073	53.10	11.31
	181	.168	22.13	26.04
	282	.194	−1.85	44.19
Pancreatic juice	23	.088	97.01	−9.09
	53	.085	95.77	−8.66
	124	.030	91.99	−4.05
	181	.041	72.03	1.19
	282	.155	43.06	15.10

[a]Concentrations of bilirubin, hemoglobin, and hematin.

[b]Lecithinase, elastase, lipase, collagenase, trypsin, amylase, and enterokinase.

Experiment IB

The capacity of the powdered pancreatic enzymes and the freshly thawed human pancreatic juice to convert hemoglobin to hematin was diminished if the solutions of the enzymes or the pancreatic juice were not utilized immediately. When the solutions were prepared and then allowed to stand for 24 hours in a refrigerator at 15°C. before being incubated with the hemoglobin solution, hematin production was markedly reduced (Table 2).

Experiment II

The enzymes lipase and elastase rapidly converted hemoglobin to hematin. The action of trypsin, lecithinase, and collagenase, though slower, proceeded to a similar end point after 22 hours of incubation. The end point was measured by the amount of hematin produced. Amylase showed little ability to convert hemoglobin to hematin even after 22 hours of incubation (Table 3).

The addition of enterokinase to the enzymes tested individually did not consistently enhance the speed or completeness of conversion of hemoglobin to hematin. The rela-

Table 2. Effect of Time on Activity of Pancreatic Enzyme and Pancreatic Juice Solutions Measured by Quantity of Hematin Produced

Type of Solution	Time (min.)	Blank (mg. %)	Pancreatic Enzymes[a] (mg. %)	Pancreatic Juice (mg. %)
Fresh	23	−9.27	−7.40	−9.09
	53	−7.45	−6.44	−8.66
	124	−4.52	11.31	−4.01
	181	−4.05	26.04	1.19
	282	−1.94	44.19	15.10
24 hrs. old	16	−7.51	−6.57	−7.76
	55	−7.84	−6.17	−7.56
	95	−5.51	−4.55	−6.88
	123	−4.83	−3.13	0.58
	158	−4.23	−3.57	−4.80
	244	−2.31	6.75	3.48
	290	−1.28	15.36	8.79

[a]Mixture contained lipase, elastase, and trypsin.

Table 3. Effect of Individual Pancreatic Enzymes on Production of Hematin from Hemoglobin

Enzyme	Time (min.)	C_b[a] (mg. %)	C_h[a] (mg. %)	C_t[a] (mg. %)
Blank	15	.049	92.6	−6.41
	178	.029	90.9	−4.96
	524	.044	90.0	−2.23
	1270	.057	73.7	6.36
Trypsin	15	.049	94.0	−5.53
	178	.030	91.6	−3.69
	524	.018	86.2	1.05
	1270	.390	−11.7	67.49
Amylase	30	.053	93.1	−4.79
	199	.041	92.4	−3.42
	535	.018	86.0	−0.26
	1285	.069	63.7	13.45
Elastase	47	.027	93.7	−5.51
	213	.102	62.3	12.56
	545	.333	− 1.95	52.47
	1300	.499	− 3.97	62.57
Lecithinase	47	.025	92.7	−5.43
	213	.068	88.6	−3.12
	545	.044	83.0	4.29
	1300	.340	−26.1	57.72
Collagenase	57	.030	92.8	−3.86
	226	.061	87.7	0.28
	557	.045	76.6	5.13
	1312	.297	−22.1	55.41
Lipase	67	.049	91.8	−2.38
	239	.300	3.9	42.74
	564	.295	− 2.03	58.77
	1322	.431	− 4.55	59.97

[a]Concentrations of bilirubin, hemoglobin, and hematin.

Table 4. Effect of Individual Pancreatic Enzymes With and Without Enterokinase on Hematin Production from Hemoglobin

Enzyme	Time (min.)	Enzyme Alone (mg. %)	Enzyme + Enterokinase (mg. %)
Blank	15	−6.41	−6.52
	178	−4.96	−4.92
	524	−2.23	−0.83
	1270	6.36	4.96
Lipase	67	−2.38	2.29
	239	42.74	39.95
	564	58.77	53.68
	1322	59.77	72.55
Elastase	47	−5.51	−4.75
	213	12.56	18.40
	545	52.47	50.24
	1300	62.57	58.31
Trypsin	15	−5.53	−4.20
	178	−3.69	−2.31
	544	1.05	2.65
	1270	67.49	50.93

tive rates of conversion of hemoglobin to hematin by the enzymes lipase, trypsin, and elastase with and without the addition of enterokinase are seen in Table 4.

Experiment IIIA

The mixture of pancreatic enzymes containing elastase, trypsin, lipase, amylase, lecithinase, and collagenase hastened the breakdown of red cells to hemoglobin. The hemoglobin was converted to hematin. Elastase, lipase, and trypsin, the enzymes most active in the conversion of hemoglobin to hematin, were also most active in lysing the red blood cells (Table 5). The production of hematin by a mixture of pancreatic enzymes was slower from red cells than from hemoglobin (Table 6).

Experiment IIIB

The individual pancreatic enzymes lipase, elastase, and trypsin, when incubated with red cells, resulted in lysis of the cells and production of hematin. The appearance of hematin was slower than during the incubation of these same enzymes with a hemoglobin solution.

Table 5. Effect of Mixtures of Pancreatic Enzymes on Production of Hematin from Red Cells

Mixture of Enzymes	Time (min.)	C_b[a] (mg. %)	C_h[a] (mg. %)	C_t[a] (mg. %)
Amylase, lecithinase, collagenase	15	.008	−64.00	−4.92
	88	.022	− 1.72	−6.15
	181	.006	0.124	−5.64
	294	−.018	− 0.74	−3.91
	404	−.011	0.22	−4.65
	532	.013	5.82	−3.45
Elastase, trypsin, lipase	15	−.028	− 0.69	−5.63
	88	−.004	0.84	−6.03
	181	−.007	15.22	−4.53
	294	−.063	119.23	−7.22
	409	.002	162.95	16.77
	532	.386	15.80	99.73
All 6	15	−.050	− 1.01	−5.88
	88	−.009	4.75	−5.68
	181	−.001	10.88	−4.18
	294	−.058	44.25	−3.21
	409	−.003	190.83	5.59
	532	−.053	129.26	39.66

[a]Concentrations of bilirubin, hemoglobin, and hematin.

Table 6. Effect of Mixture of Pancreatic Enzymes With and Without Enterokinase on Hematin Production from Red Blood Cells

Time (min.)	Pancreatic Enzymes (mg. %)	Pancreatic Enzymes + Enterokinase (mg. %)
15	—	−5.88
23	−7.40	—
53	−6.44	—
88	—	−5.68
124	11.31	—
181	26.04	−4.18
282	44.19	—
294	—	−3.21
409	—	5.59
532	—	39.66

DISCUSSION

Hematin is one of the degradation products of hemoglobin, and its production from hemoglobin in vitro is augmented by a mixture of pancreatic enzymes. The pancreatic enzymes which most enhanced the rate of conversion of hemoglobin to hematin were lipase, elas-

tase, and trypsin. The same enzymes were also most active in hematin production from red cells. Our in-vitro studies support Northam's contention that pancreatic enzymes in vivo hasten the production of hematin from red cells within the interstitium of the pancreas and peritoneal cavity [7]. Thus the appearance of hematin in the presence of haptoglobin in the serum of patients with pancreatitis indicates the hemorrhagic form of the disease. It is possible that other factors in addition to pancreatic enzymes, such as bacteria, may participate in the conversion of hemoglobin to hematin; our preliminary work indicated that *Streptococcus faecalis* and *Clostridium perfringens* can speed the conversion of hemoglobin to hematin.

Hematin itself may contribute to the pathogenesis of pancreatitis. A marked reduction in blood volume often accompanies acute pancreatitis and not infrequently leads to marked degrees of hemoconcentration and dehydration [3]. The sequelae of dehydration and hypovolemia in pancreatitis are often oliguria and renal failure [4]. Clearance of serum hematin by the kidney under conditions of dehydration and low urinary flow brings hematin in contact with the renal tubules for sufficiently long periods that damage to the tubules results [6, 10]. Production of large amounts of hematin in the peritoneal cavity during acute pancreatitis may contribute to the development of renal failure.

Factors affecting the serum levels of hematin are the amount of pancreatic and peritoneal hemorrhage, the presence of pancreatic enzymes and perhaps bacteria in the interstitium of the pancreas and peritoneal cavity, and the rate of clearance of serum hematin by the kidneys.

SUMMARY

Production of hematin from hemoglobin in vitro is augmented by a mixture of powdered pancreatic enzymes and human pancreatic juice. Those enzymes which most affected the rate of conversion of hemoglobin to hematin were lipase, elastase, and trypsin. These same enzymes were also most active in hematin production from red cells.

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