A Further Inquiry into the Enzymatic Digestion of Blood *

PAUL NEMIR, JR., M.D., S. J. MUCHA, M.D., H. R. HAWTHORNE, M.D., D. L. DRABKIN, M.D.

From the Departments of Surgery and Biochemistry and the Harrison Department of Surgical Research, Graduate School of Medicine, University of Pennsylvania

IN A PREVIOUS communication we reported the finding of an abnormal hemin pigment not previously reported which was formed in the gut lumen of dogs having strangulation intestinal obstruction.[12, 13] This pigment subsequently appeared in the peritoneal fluid and finally in the blood stream, thus aiding in our understanding of the pathogenesis in this disease. A similar pigment has been found clinically in one instance in association with gangrenous intestine.[5]

It was possible to obtain *in vitro* a solution having similar spectrophotometric characteristics by the admixture of activated pancreatic juice and whole blood.[14] In addition to revealing the spectrophotometric characteristics, this fluid was highly lethal on intravenous injection.[15] Moreover, small amounts of this admixture, when injected into the obstructed pancreatic duct of the dog, invariably resulted in the death of the animal with a picture entirely similar to that seen clinically in hemorrhagic necrotizing pancreatitis.[16]

The importance of the vascular component in the pathogenesis of acute necrotizing pancreatitis was stressed by these studies. It was postulated that as a result of ischemia and vessel wall damage incident to vascular compromise, blood was allowed to come into contact with pancreatic juice in the gland parenchyma and an extremely toxic substance was then formed.

More recently, Lillehei [9, 10] reported the findings of a progressive rise in plasma hemoglobin levels and the appearance of an abnormal hemin pigment, similar to that described by us in the plasma of dogs dying of irreversible hemorrhagic shock. Both findings were felt to be related to mucosal necrosis of the bowel occurring as a result of prolonged hemorrhagic shock. At autopsy, these dogs had severe mucosal congestion and necrosis of the small bowel. The lumen of the bowel was filled with dark bloody fluid, a picture noted by many others in irreversible shock,[22] and similar to the local picture in strangulation intestinal obstruction.[12] A constant finding in all the animals dying of irreversible hemorrhagic shock was increasing opacity of the plasma in the period following retransfusion. He concluded that a rising plasma hemoglobin along with the appearance of an abnormal hemin pigment was a reliable sign of bowel necrosis and of impending death in dogs following a prolonged period of hemorrhagic shock.

With the finding of the unusual pigment in strangulation intestinal obstruction and irreversible shock and its implication in acute hemorrhagic necrotizing pancreatitis, the possibility of some common denominator in these three conditions became apparent. In each instance blood and enzymes came into contact with each other. A further inquiry into the enzymatic digestion

* Presented before the American Surgical Association, San Francisco, Calif., April 15–17, 1959.

Aided by Grants from the A. Atwater Kent Fund, Grant No. H1282(C)4 from the National Heart Institute of the National Institutes of Health, Public Health Service, and from the Squibb Institute for Medical Research.

of blood was therefore undertaken. The results of this study detailed below were obtained after more than 120 *in vitro* experiments and over 75 experiments on the relationship between bacterial content, spectrophotometric abnormality, and toxicity.

Reproduction of the Abnormal Hemin Pigment in Vitro

Since the blood which entered the strangulated gut in the original experiments was irreversibly altered by some component or combination of components within the gut, attempts at reproduction of the pigment were made by incubating blood with pure and activated pancreatic juice, bile, succus entericus, macerated mucosa, and the usual bacterial organisms found in the intestinal tract. Since E. coli, hemo. Cl. Welchii, and hemo. streptococci were invariably found in the earlier experiments these were the organisms used in this study. Previous communications have dealt in detail with this phase of the problem,[14] but the continuing experiments have developed some new aspects, and have necessitated the emphasis of certain points.

The hemin pigment may be obtained by an admixture of blood and activated pancreatic juice. The reactions which result in its production are specific and complex.

When two parts of activated pancreatic juice are mixed with one part of whole blood and then incubated 24 to 36 hours, a mixture which will yield the abnormal hemin pigment is obtained. A number of factors must be carefully considered, however, if the pigment is to be uniformly obtained:

(1) Factors relating to the pancreatic juice: Meat stimulated juice has been invariably used in these experiments. There is good evidence[9] that meat stimulated juice is higher in enzymatic activity than hydrochloric acid stimulated juice. There is further evidence in our studies that the thick viscid juice obtained with meat stimulation is a much more effective substance in the mixture than is the hydrochloric acid juice. Time factors are important in dealing with pancreatic juice. The pure juice may be kept refrigerated at 4° C. for 48 to 72 hours without an appreciable loss in enzymatic activity. On the other hand, once it is activated or, at least, once it comes into contact with succus entericus, it begins to decrease in enzymatic activity in a few hours. We have invariably used the juice immediately. Moreover, there is good evidence that the juice cannot be frozen and then reconstituted without loss of enzymatic activity. Another factor of importance here is that the pure pancreatic juice is not entirely sterile. While the colony count is extremely low, the usual gastro-intestinal bacterial flora will be present. The pancreatic juice is collected in iced sterile containers and used immediately.

(2) Factors relating to activation of the juice: This is the most difficult and variable factor with which we have had to deal. Activation of the juice may be quite simply carried out by the addition of succus entericus, collected from animals with Thomas cannulae, to the pure pancreatic juice and allowing the mixture to stand at room temperature for two hours or in the refrigerator at 4° C. for four hours. The activation obtained in this manner has been most satisfactory. We have generally used one part of succus to fifteen to twenty parts of pure juice. If succus is used, however, other variables must be taken into consideration. The succus is invariably heavily contaminated with organisms. While only a few colonies may be obtained by the plate method immediately after collection, these are increased many times following incubation. With the succus as the activator, therefore, the presence of large numbers of bacterial organisms has complicated the picture.

The whole problem of activation of pancreatic juice is a complex one. It is, of course, well known that the juice may be

activated by bacteria or by simply being allowed to stand for a length of time. There is good evidence in our studies, however, that activation is much more complete with succus or with active enterokinase. By using enterokinase it is possible to markedly decrease the amount of contamination. However, its disadvantage lies in the fact that there has been a good deal of variability in the ability of purified enterokinase which we have used to activate the pancreatic juice. The degree of activation is of importance in the completeness of the enzymatic digestion of blood.

In our studies we have used succus almost entirely as the activator. Our purpose in doing this has been twofold. First, it is the most potent activator and its performance is consistent. Second, it simulates more closely the activation of the juice in vivo in the intestinal tract. The succus is filtered through a Whatman #40 paper filter to remove any gross particles and immediately mixed with the pancreatic juice.

(3) Factors relating to the blood: There is evidence that the ratio of activated pancreatic juice to blood must be at least two to one if the abnormal pigment is to be consistently obtained on spectrophotometric examination. Secondly, it is well to note the very small quantities of blood used in lethal mixtures. For example, in the experiments in which the blood-activated juice mixture was injected into the obstructed pancreatic duct, the amount of blood used was never more than 2 ml.[16] In the intravenous injections amounts of blood as low as 5 ml. were lethal following incubation with activated pancreatic juice. Of more importance relating to this factor, is that there is evidence that the blood must be hemolyzed before digestion of the hemoglobin by the enzymes can occur. Further reference will be made to this subsequently.

(4) Factors relating to bacterial content: In all our previous studies it has not been possible to fully assess the role of the bacterial organisms or their end products in the pathogenesis of strangulation obstruction or of hemorrhagic necrotizing pancreatitis. This is also true of irreversible shock.[11, 21] It is generally agreed that their role is an important one since administration of antibiotics appreciably alters the course of all three of these diseases, at least in the experimental animal.[2-4, 6-8, 19, 20] With respect to strangulation intestinal obstruction we felt that antibiotics exerted a protective action on the mucosal lining of the bowel by delaying the destruction by the bacterial organisms and thereby lengthening the time at which the gut wall became permeable to its intraluminal contents.[17] Another mechanism of action of the bacteria, however, appears to be uncovered in more recent studies.

In the numerous experiments to reproduce the pigment it was found that uniform results were not obtained with the same mixtures. For example, sometimes the pigment would be obtained from a mixture of blood and pancreatic juice which had not been specifically activated with succus or enterokinase. At other times the pigment would not be obtained with this same mixture. Also, pure crystalline trypsin sometimes yielded an unusual hemin pigment on incubation with blood, but more frequently did not. Originally this variability was thought to be explainable on the basis of concentration.

When these studies were recently repeated, carefully prepared sterile crystalline trypsin and chymotrypsin failed to yield the pigment changes when mixed with sterile blood. This suggested the possibility that it was necessary for the blood to be hemolyzed in order for the trypsin to exert its effect. Crystalline trypsin in the same amount was then incubated with hemolyzed red blood cells (one part of red cells, one part toluene, one part sterile water refrigerated at 4° C. for 18 to 24 hours) and yielded characteristic alterations in spectra. The same was true of pure crystalline chymotrypsin (Fig. 1).

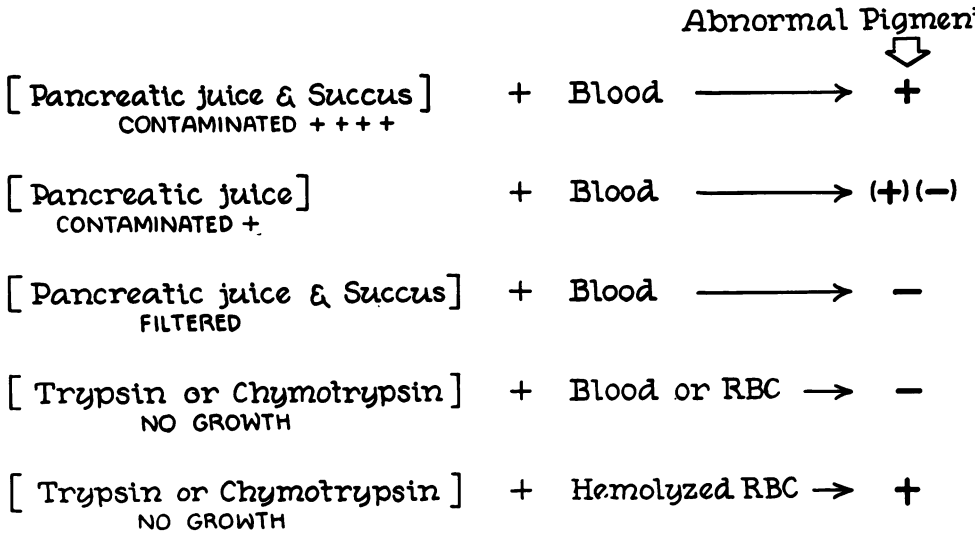

FIGURE 1

When pure crystalline trypsin or chymotrypsin are used a great deal of pigment precipitates. Our analysis is on the material still in solution. It should be pointed out that the absorption spectra of the soluble hemin pigments obtained by means of trypsin and chymotrypsin are qualitatively different than those obtained by means of pancreatic juice and succus entericus. Only occasionally in the past have we obtained curves with the pancreatic juice and succus similar to those now obtained with trypsin and chymotrypsin. It is probable that progressive alterations in spectra may occur under the enzymic digestive conditions which have been employed, and that the pigments observed with tryptic or chymotrypic digestion may represent degradation beyond the point obtained with the succus activated pancreatic juice. We have some suggestive evidence which appears to favor this view (Fig. 2).

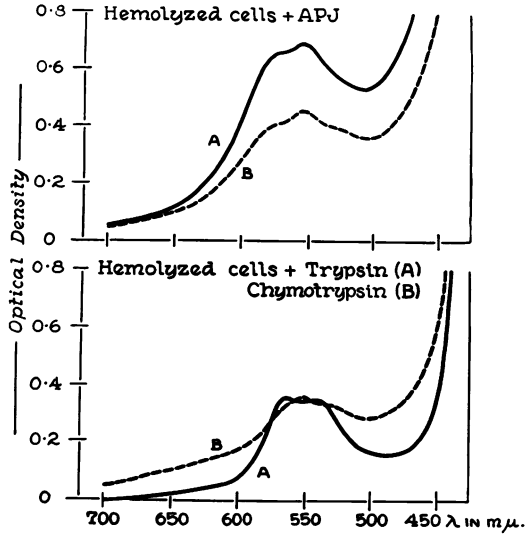

FIG. 2. Upper Graph: A and B represent separate experiments. In each the spectra were obtained after treatment with the reducing agent $Na_2S_2O_4$. In A note that the maximum at 575 mμ is lower than that at 554 mμ. In B note an additional maximum at 530 mμ. Lower Graph: In A note that maximum at 575 mμ is slightly higher than that at 554 mμ. In B note shift of maximum to 550 and 530 mμ.

Careful review of all our previous data regarding the pigment was undertaken and yielded the following pertinent points:

(1) When the heavily contaminated succus-activated pancreatic juice was passed through a Zeitz or Berkfeld filter and then incubated with blood, the abnormal hemin pigment was not obtained and the mixture was not lethal. This was attributed to the fact that the enzymes were precipitated out on the filter along with the removal of the

Dogs	Material injected	Amount (ml./kg.)	Died	Average survival	Spectrophotog.	Bact. growth
3	APJ	1·5-4·0	...	...	...	marked
3	blood or RBC	5·5-7·0	...	...	–	sterile
2	blood + P.J.	1·5-2·0	...	...	all +	marked
7	blood + APJ	1·0-6·3	6	7 hr.	all +	marked
4	plasma + APJ	1·5-6·6	4	10 hr.	...	marked
3	RBC + APJ	2·8-4·4	3	4 hr.	2 + 1 –	marked
6	blood+trypsin	2·0-2·5	1	16 hr.	all –	marked
3	blood, plasma or RBC + APJ (FILTERED)	1·0-2·2	...	...	–	sterile

FIGURE 3

bacteria and that, therefore, the enzymatic digestion of the blood did not occur.

(2) In every instance where cultures had been taken on the mixtures which did yield the pigment, a hemolytic organism—either hemolytic Clostridia, hemolytic streptococci and/or hemolytic B. coli—was present.

Relationship Between Toxicity, Bacterial Content, and Spectrophotometric Abnormality

Evaluation of the toxicity of a given material cannot generally be measured in precise terms. Particularly is this true in this study due to the complexity of the substances being evaluated. To obtain an LD 50 using dogs is not very satisfactory, and if mice are used the additional factor of species difference is introduced.

Injection studies have, however, demonstrated unequivocally the extreme toxicity of the material resulting from the enzymatic digestion of blood as prepared by us.

The methods used have been previously reported. Briefly, pancreatic juice is collected under ice directly from the major pancreatic duct through a Thomas cannula. Succus entericus is also obtained through the cannula. The succus is filtered through a Whatman #40 paper filter to remove gross particles and then added to the pure juice—one part succus to fifteen to tweny parts pancreatic juice. This mixture is allowed to stand for two hours at room temperature or four hours in a refrigerator at 4° C. It is then mixed with blood, red blood cells, or plasma in two to one proportions. This mixture is incubated at 37.5° C. for twenty-four to thirty-six hours, and is then immediately used. Intravenous injections are given slowly into a leg vein. Injections into the major duct are administered as previously reported.[16] Spectrophotometric and bacteriologic studies are routinely taken.

Results are shown in Figure 3. Intravenous injection of the activated pancreatic juice alone, or the blood or red cells alone after 36 to 48 hours of incubation was without appreciable effect. This was true also of filtered juice-blood, plasma, or red cell mixture. Note also that the filtered juice-blood, or filtered juice-red cell mixtures did not yield the hemin pigment. While this may in part be due to the decrease in enzymatic activity following passage through a Zeitz or Berkfeld filter, it is more likely, in the light of recent experiments, that the absence of hemolytic organisms was the crit-

ical factor; i.e., without the bacteria being present to hemolyze the cells, the enzymes were unable to act on the hemoglobin pigment.

Note that a mixture of blood and pure juice (not activated with succus or enterokinase) also yielded the hemin pigment. This is not particularly remarkable since bacteria were present in the mixture and it is well known that the juice may be activated by organisms or by simply standing for a sufficient length of time. This fluid was not lethal. It may be that the total amount administered was insufficient to cause death, since it was less than the average in experiments in which blood, plasma, or red cells were incubated with succus activated juice, but another likely explanation is that the enzymatic digestion of the blood was incomplete due to the incomplete activation of the pancreatic juice.

Note that 13 of the 14 animals receiving a mixture of activated juice with blood, red blood cells, or plasma died. The average length of survival for the animals receiving the activated juice-blood mixture was seven hours; for the activated juice-plasma mixture, ten hours; and for the activated juice-red cell mixture, four hours.

Note that in one instance the activated juice-red cell mixture failed to reveal the hemin pigment, but was nonetheless extremely toxic.

While it was possible to obtain the pigment when 50 mg. of trypsin was added to 2 ml. of blood, the pigment was not obtained when greater quantities of blood were used with 50 mg. of trypsin. One of the six animals receiving blood and trypsin (2.2 ml./kilo. containing a total of 50 mg. of trypsin) died.

Discussion

Except when the Zeitz or Berkfeld filtered pancreatic juice was used, or when the blood or red cells alone was used, the injected fluids were invariably contaminated with organisms. Qualitatively, B. proteus, hemolytic or non-hemolytic streptococci, hemolytic or non-hemolytic Clostridia, hemolytic or non-hemolytic B. coli, and occasionally B. subtilis and diphtheroids were the organisms found to be present. One or more of these were invariably present, and, of greater significance, one or more of the hemolytic organisms was invariably present. In some but not all of the cases quantitative bacterial counts were done.[15] The colony count markedly increased following incubation at 37.5° C. in all instances. This was particularly true when blood, plasma, or red cells were in the mixture, since these substances are an excellent substrate for bacterial growth.

While it was obvious from the foregoing that a mixture of activated pancreatic juice with blood, plasma, or red cells was extremely lethal in small amounts, it was difficult to assess the precise role of the bacterial factor.

We feel the more recent studies have greatly clarified this problem. As stated, pure crystalline trypsin or chymotrypsin when mixed with sterile whole blood or red blood cells failed to yield the pigment. If, however, the red cells were hemolyzed and then incubated with trypsin or chymotrypsin, the abnormal pigments were demonstrated indicating that the enzymes were unable to act upon the hemoglobin as long as the red cell was intact. The question of the identity of the pigments produced by means of trypsin and chymotrypsin with that found with succus activated pancreatic juice and blood remains unsettled at present.

It is to be realized that the admixture of Zeitz or Berkfeld filtered pancreatic juice with whole blood or red blood cells also failed to yield the pigment. The mixture of millipore filtered pancreatic juice to hemolyzed red blood cells did yield the pigment. The only apparent difference between the bacteria-laden activated pancreatic juice-blood mixture and the bacteria-free activated pancreatic juice-hemolyzed red cell

mixture is the absence of the extremely foul odor in the latter.

These studies more clearly define the role of the bacteria. It would appear that at least one important function of the organisms is to hemolyze the blood if enzymatic digestion of the hemoglobin is to occur. Certain strains of hemolytic streptococci and Clostridia may begin to hemolyze blood within less than two hours. An increase in the plasma hemoglobin occurs in experimental strangulation intestinal obstruction,[1,12] acute necrotizing pancreatitis,[1] and irreversible shock. If this explanation be true, then it would help to explain the beneficial effects of antibiotics in strangulation obstruction,[2-4] acute pancreatitis [8, 19, 20] and ischemic [7] or hemorrhagic shock.[6]

Further delineation of the role of the pigment is, however, necessary. The fact remains that in the injection experiments the activated pancreatic juice-plasma mixture was also lethal. It may be that the incomplete digestion of plasma proteins also yields a lethal mixture. With the anticipated ability to separate the bacteria from all of the mixtures by the use of the millipore filter, it should also be possible to clarify this point.

Summary and Conclusions

The enzymatic digestion of blood by activated pancreatic juice under the conditions of our study yields an extremely lethal substance. The digestion of blood by pancreatic enzymes also yields altered hemin pigments which have not been previously identified *in vivo*. The finding of presumably related pigments in experimental strangulation obstruction, and in irreversible shock; and their implication in necrotizing pancreatitis has indicated some common denominator in these three conditions. Such pigment derivatives of hemoglobin may in themselves be toxic or they may simply serve as an indicator of the enzymatic digestion of blood with the formation of some as yet unknown toxic substance.

In each of the three conditions blood may be acted upon by pancreatic enzymes. This occurs in the bowel lumen in intestinal obstruction and in the pancreatic parenchyma in necrotizing pancreatitis. Bowel ischemia with mucosal necrosis in irreversible shock presents a similar situation to that seen in strangulation intestinal obstruction. A rising plasma hemoglobin is also an invariable accompaniment, indicating hemolysis.

Further clarification of the important role of the bacterial organisms is afforded by these studies. There has always been much controversy in these three conditions with respect to whether the bacterial factor or some toxic factor is the most important.

We advance the concept that, in the experimental animal at least, both factors are necessary. The bacterial organisms are necessary to hemolyze the blood and then a toxic substance is formed by the enzymatic digestion of hemoglobin or other proteins.

Acknowledgments

We wish to acknowledge our gratitude to Dr. Frank P. Brooks, Associate Professor of Physiology, for supplying us with pancreatic juice from his animals and for his helpful advice. Our thanks are due also to Dr. M. H. L. Friedman, Professor of Physiology at the Jefferson Medical College, for supplying us with enterokinase, and to Mr. Steven Horoshak of the National Drug Company for supplying us with the pure crystalline trypsin and chymotrypsin.

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