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Mitochondrial Dysfunction Induced by Pancreatitis-Associated Ascitic Fluid (41580)

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Abstract. Acute hemorrhagic pancreatitis (AHP) involves multiple organ failure probably caused by the toxic factor(s) released in pancreatitis-associated ascitic fluid (PAAF). We found that PAAF interferes with hepatic mitochondrial respiration resulting in severe disturbances in respiratory control (RCR) and ADP/O ratios. Pancreatitis was induced in dogs by retrograde pancreatic duct infusion and the resultant PAAF was centrifuged, filtered, and frozen until used. Two human PAAFs collected from AHP patients were treated in a similar manner. Rat liver mitochondrial oxygen uptake was measured at 30°C before and after addition of ADP and PAAF. Paired control runs were made using pooled heat-inactivated dog serum. Tests with nine canine PAAFs showed a mean increase of 120% in state 4 respiration ($P < 0.0001$). After exposure to PAAF, addition of ADP to previously coupled mitochondria did not induce state 3 respiration. The human PAAFs both showed significant increases in state 4 respiration ($P < 0.01$) and a marked decrease in RCR. Dose-response tests with human and canine PAAFs showed a positive correlation between percentage increase in state 4 respiration and the concentration of PAAF used. These results confirm the presence in PAAF of mitotoxic substance(s) which cause irreversible mitochondrial damage. Inhibition of coupled mitochondrial respiration by PAAF with the resultant fall in ATP may be the causative agent for the tissue and organ damage observed in AHP.

Acute pancreatitis has widespread systemic effects often resulting in multiple organ failure (1). The serious cardiac (2), hepatic (3), renal (4), and respiratory (5) effects of hemorrhagic pancreatitis have led many investigators to postulate the presence of a systemic toxic factor(s) in the ascitic fluid which accumulates during the disease. It has been shown that the injection of pancreatitis-associated ascitic fluid (PAAF) into healthy dogs causes a transient hypotension (6) and that ip injections of PAAF in rats caused a significant increase in hematocrit (7). As early as 1966, Rodgers and Carey (8) showed that peritoneal lavage of dogs with experimental pancreatitis was beneficial. Later investigators demonstrated that similar procedures in humans with acute hemorrhagic pancreatitis (AHP) improved their condition. These results have been interpreted to indicate that the peritoneal lavage either removed or diluted a toxic factor in the ascitic fluid, resulting in improvement in the patient (9).

Pappas *et al.* (10) recently demonstrated that PAAF samples, obtained from a canine model of AHP, caused a decrease in rat liver cell oxygen consumption. This occurs in a dose-dependent fashion and correlates well with the degree of pancreatitis induced in the experimental dogs from which the PAAF was obtained. The metabolic depression observed in liver cells after exposure to PAAF, could be used to explain the liver dysfunction observed in severe pancreatitis (3). Since the toxic factor(s) present in PAAF depress cellular metabolism, this may be responsible for the multiple organ failure found in AHP.

Mitochondria are the primary organelles of cellular respiration. If the toxic factors in PAAF have an intracellular effect which results in metabolic depression, it is probable that PAAF has a direct effect on mitochondrial respiratory activity. The present study tests the effects of PAAF (both canine and human) on the respiratory activity of rat liver mitochondria. We used the respiratory control and ADP/O ratios as indicators of mitochondrial activity (11) in order to develop a sensitive assay for the toxicity of dog and human PAAF samples.

Methods. Experimental canine pancreatitis was induced by retrograde infusion of a trypsin–sodium taurocholate solution into the cannulated pancreatic duct of dogs (7). The resultant PAAF was drained from the abdominal cavity into sterile plastic containers and centrifuged to remove debris. The fluid was then passed through a 0.2-μm Millipore filter

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and stored frozen until used. Osmolarities were tested using a vapor pressure osmometer (Wescor 5100) and found to range between 265–375 mOsm. Aerobic and anaerobic plates of the −20°C stored PAAFs were done to check for bacterial contamination and any samples that showed growth were discarded. Endotoxin tests performed on 1:100 diluted PAAF samples (Pyrogent, Mallincdrodt, St. Louis, Mo.), were negative. Human PAAFs collected during abdominal surgery were filtered, tested, and stored as described above.

Mitochondrial preparation. Healthy Sprague–Dawley female rats (220–250 g) were decapitated, exsanguinated, and the liver was removed rapidly and placed in ice-cold 0.25 *M* sucrose. All preparatory steps were carried out at 4°C. After carefully removing the larger blood vessels and fatty tissue, the liver was minced with a scissors, washed twice, then resuspended (1:4) in 0.25 *M* sucrose. The liver mince was dispersed by five passes with a loose Teflon–glass homogenizer. The cell suspension in 0.25 *M* sucrose–0.004 *M* Tris (pH 7.2) was then transferred to a homogenizer tube and after 10 passes with a tight Teflon pestle, the homogenate was poured into 50-ml polycarbonate tubes and spun at 400*g* for 5 min in a refrigerated centrifuge. The resultant supernate was carefully decanted and recentrifuged at 10,000*g* for 10 min. After discarding supernate, fluffy coat, and red cells, the greyish-brown mitochondrial pellet was resuspended in 8 ml of 0.25 *M* sucrose. The protein content of the mitochondrial suspension was determined by the Biuret reaction using dilutions of a weighed sample of bovine serum albumin to construct the calibration curve (12).

Mitochondrial oxidative activity. Mitochondrial activity was determined in a glutamate–malate medium of the following composition: 0.005 *M* K-phosphate buffer; 0.07 *M* KCl; 0.005 *M* glutamate; 0.005 *M* malate in 0.15 *M* sucrose–0.04 *M* Tris (pH 7.2). Mitochondrial oxidative activity was measured at 30°C using a micromodification of the YSI Model 53 Biological Oxygen Monitor as described by Lessler and Scoles (13). The glutamate–malate medium (1.8 ml) was added to a magnetically stirred, temperature-controlled reaction vessel, and allowed to equilibrate with air for a period of 5 min. Following equilibration, a 0.2-ml sample of 10 to 15 mg/ml of a mitochondrial preparation was added to the stirred aerated medium giving a final concentration of 1 to 1.5 mg of mitochondrial protein/ml.

The oxygen uptake was monitored with an (YSI 5331) oxygen electrode for a period of 3 min, after which 3 μl of 0.1 *M* ADP (Sigma, A-8146) was added to the chamber to induce state 3 respiration. Two minutes after the oxygen trace returned to state 4 respiration, 20 μl of a PAAF sample (final conc. 1:100) was introduced into the reaction vessel and the oxygen uptake record continued for an additional 3 min, then 3 μl of 0.1 *M* ADP again was added to the reaction chamber and the oxygen trace continued. Identical control runs were made using pooled, heat-inactivated (56°C, 1 hr) dog serum (PSI) in place of the PAAF. Dose response was monitored by studying unstimulated (state 4) mitochondrial respiration for 5 min then adding 5, 10, 20, 40, or 80 μl of PAAF (final dilution 1:400, 1:200, 1:100, 1:50, 1:25). The oxygen uptake measurements were continued for an additional 3 min after which 3 μl of 0.1 *M* ADP was added to see if state 3 respiration could be reinduced (See Fig. 1).

All data are reported as mean ± standard deviation and were tested for significance by *t* test. Curves were derived from a linear regression program.

Results. Each of the PAAF samples was tested to determine osmolarity, pH, bacterial contamination, and presence of endotoxin. The pH of the PAAF samples was approximately that of plasma and the PAAF used in the tests was adequately buffered to 7.2 by the mitochondrial medium used. Osmolarity of the canine PAAFs, as measured with a vapor pressure osmometer, ranged from 295–335 mOsm and that of the two human PAAFs were 265 and 375 mOsm. Aerobic and anaerobic cultures were run on the stored PAAFs to insure sterility and contaminated samples were discarded. After defrosting, experimental PAAFs were diluted to 1:100 and tested for the presence of endotoxin, using the Limulus Amebocyte Lysate test, and found to be negative.

Several different substances including dog plasma, heat-inactivated dog plasma (56°C, 1 hr), dog serum, heat-inactivated dog serum

TABLE I. MITOCHONDRIAL METABOLISM BEFORE AND AFTER EXPOSURE TO 20 μl OF CANINE PAAF OR PSI

	St. 4 (nmol O_2/min/mg)			RCR		ADP/O	
PAAF	Bef. add.	Aft. add.	% In.	Bef. add.	Aft. add.	Bef. add.	Aft. add.
1	19.7 ± 1.6	30.8 ± 2.5	56*	3.0 ± 0.2	—[a]	1.7 ± 0.3	—[a]
2	16.2 ± 4.5	26.4 ± 9.2	69*	3.7 ± 0.2	—	2.5 ± 0.1	—
3	20.6 ± 3.4	39.4 ± 7.8	91*	2.7 ± 0.2	—	1.9 ± 0.2	
4	11.4 ± 2.4	23.7 ± 8.0	108*	3.1 ± 0.7	—	1.9 ± 0.2	—
5	9.5 ± 1.6	23.3 ± 4.0	145*	3.0 ± 0.1	—	2.4 ± 0.4	—
6	10.6 ± 1.9	26.0 ± 4.0	146*	4.0 ± 0.1	—	2.1 ± 0.3	—
7	17.7 ± 2.7	43.6 ± 7.2	146*	3.9 ± 0.6	—	1.9 ± 0.4	—
8	12.7 ± 1.9	32.3 ± 3.2	154*	4.2 ± 0.6	—	2.0 ± 0.1	—
9	17.6 ± 0.8	45.6 ± 9.6	159*	3.2 ± 0.2	—	2.0 ± 0.2	—
Mean[b]	15.0 ± 4.7	33.1 ± 9.8	120*	3.4 ± 0.7	—	2.0 ± 0.3	—
Control[c]	15.2 ± 5.1	17.3 ± 5.8	14	3.3 ± 0.6	2.0 ± 0.4	2.0 ± 0.4	1.8 ± 0.2

[a] ADP addition after PAAF resulted in decreased respiration. Ratio was not calculated.
[b] Mean of 36 determinations (4/PAAF). Data arranged in order of percentage increase in state 4 respiration, mean ± SD.
[c] Mean of 36 paired control runs with pooled heat-inactivated dog serum (PSI).
* State 4 increase statistically significant ($P < 0.01$).

(PSI), 1% serum albumin, rat serum, and heat-inactivated rat serum were tested in a search for a control substance with minimal effects on rat liver mitochondria. Small additions of any of these substances at a 1:100 dilution caused some change in mitochondrial oxidative activity. PSI was chosen as the control substance by virtue of its stability when stored frozen, and its minimal effects on the oxidative activity of the mitochondria in our test system.

Mitochondria exposed to canine PAAF showed a significant increase ($P < 0.0001$) in state 4 respiration of 120%. Paired control runs performed with PSI showed a mean increase in state 4 respiration of only 14%. Although PAAF samples and PSI both caused stimulation of state 4 respiration, the increase due to PAAF samples was more sustained and approximately nine times greater than that observed with PSI (Table I).

Mean RCR values of our mitochondrial preparations before exposure to PAAF (Table I) were 3.4 ± 0.7. After addition of PAAF, there was a dramatic increase in state 4 respiration and further addition of ADP resulted in decreased respiratory activity making it impossible to calculate a post-PAAF RCR. Similar tests with paired controls gave an RCR of 3.3 ± 0.6 before PSI addition and 2.0 ± 0.4 after incubation with PSI.

The mean ADP/O ratio of mitochondrial samples before the addition of PAAF averaged 2.0 ± 0.3 ($n = 36$), after exposure to PAAF these mitochondrial preparations, without exception, showed stimulated state 4 respiration and subsequent addition of ADP actually resulted in an inhibition of this enhanced rate. Control runs performed with PSI showed no significant change in this parameter, mean control ADP/O was 2.0 ± 0.4 ($n = 36$) before addition of PSI and 1.8 ± 0.2 after addition (Table I).

Dose response tests performed with six different canine PAAFs at concentrations of 5, 10, 20, and 40 μl showed a positive correlation between percentage increase in state 4 respiration and dosage of PAAF used (see Fig. 2). The two human PAAFs were tested at concentrations of 10, 20, 40, and 80 μl and showed a positive correlation between percentage increase in state 4 respiration and dosage of PAAF used (see Fig. 3).

Tests of the two human PAAFs showed a mean increase of 26% in state 4 respiration as compared to paired control runs with PSI (Table II). As was seen with the canine PAAF tests, both human PAAFs caused an increase in state 4 respiration. Mean RCR of liver mitochondria was 3.4 ± 0.7 before and 1.8 ± 0.3 after incubation with human PAAF. The observed 47% decrease in RCR was significant at the $P < 0.001$ level. Paired control runs gave RCRs of 3.1 ± 0.3 before and 2.3 ± 0.4 after PSI incubation. Rat liver mitochondria exposed to human PAAF had a mean ADP/O

TABLE II. MITOCHONDRIAL METABOLISM BEFORE AND AFTER EXPOSURE TO 20 μl OF HUMAN PAAF OR PSI

	St. 4 (nmol O_2/min/mg)			RCR			ADP/O	
PAAF	Bef. add.	Aft. add.	% In.	Bef. add.	Aft. add.	Dif.	Bef. add.	Aft. add.
hp1	20.7 ± 4.2	26.9 ± 3.2	30*	3.5 ± 0.5	1.7 ± 0.3	1.8	1.8 ± 0.2	—[a]
hp2	21.1 ± 3.1	25.9 ± 3.3	23*	3.3 ± 0.7	1.9 ± 0.3	1.4	1.9 ± 0.2	—
Mean[b]	20.9 ± 3.6	26.4 ± 3.3	26*	3.4 ± 0.7	1.8 ± 0.3	1.6	1.8 ± 0.2	—
Control[c]	22.0 ± 3.8	24.0 ± 5.1	9	3.1 ± 0.3	2.3 ± 0.4	0.8	1.7 ± 0.2	1.9 ± 0.2

[a] ADP addition after PAAF did not induce state 4 respiration.
[b] Mean of eight determinations with two human PAAFs (4/PAAF), mean ± SD.
[c] Mean of eight paired control runs with heat-inactivated dog serum (PSI).
* State 4 increase statistically significant ($P < 0.01$).

ratio of 1.8 ± 0.2 before addition of human PAAF, and did not return to state 4 respiration after addition of ADP making it impossible to calculate the ADP/O ratio. Paired PSI controls showed no significant change in ADP/O ratios with mean values of 1.7 ± 0.2 before PSI addition and 1.9 ± 0.2 after (Table II).

Discussion. Patients suffering from acute pancreatitis often manifest some form of liver dysfunction (3). Kitamura *et al.* (14) found a 40% reduction in rabbit hepatic mitochondrial ATP synthesis 2 days after inducing experimental pancreatitis. They interpreted these alterations in mitochondrial metabolism as being due to some toxic factor released during the acute pancreatitis. Our data show that PAAF significantly increases state 4 respiration and interferes with the respiratory con-

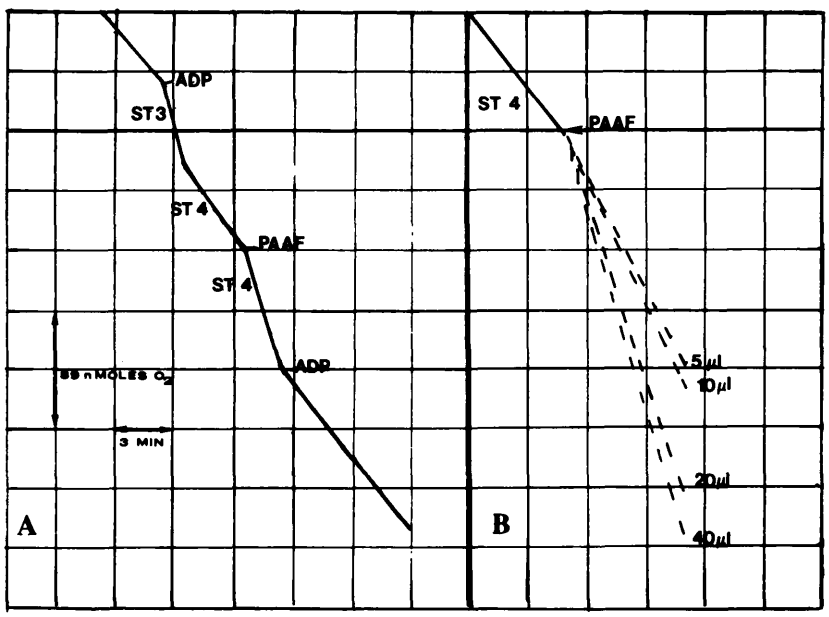

FIG. 1. (A) Actual record of a typical experimental test with canine PAAF. Unstimulated state 4 respiration of liver mitochondria was 18 nmole O_2/min/mg and on addition PAAF (1:100) oxygen consumption rose to 38 nmole O_2/min/mg, a 111% increase due to the uncoupling action of the PAAF. After exposure to PAAF, the addition of ADP resulted in an inhibition of the enhanced state 4 metabolic rate. (B) Summary dose-response curve generated by comparing average increases in state 4 respiration induced by different concentrations of canine PAAF. Mitochondrial respiration on exposure to 5 μl of PAAF increased 55%, 10 μl of PAAF increased 70%, 20 μl of PAAF increased 145%, and 40 μl of PAAF increased 175%.

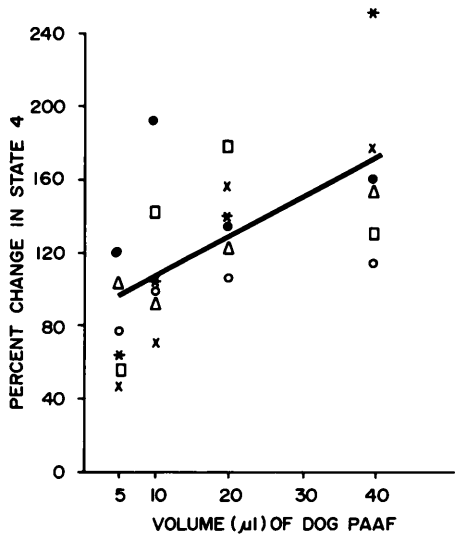

FIG. 2. Dose-response curve for six different canine PAAFs (as indicated by the six different symbols used). The solid line was generated by linear regression.

trol of hepatic mitochondria (Tables I, II). At low concentrations, PAAF causes a disturbance in the ability of previously coupled mitochondria to utilize ADP immediately after exposure to PAAF. There is a growing body of evidence that suggests that the toxic factor(s) present in PAAF interferes with oxidative phosphorylation and ATP production in liver mitochondria. In our experiments (see Fig. 1), after exposure to PAAF, the mitochondria were unable to return to state 3 respiration on further addition of ADP, or showed a marked decrease in RCR. These effects were seen at PAAF dilutions of 1:100 demonstrating that the mitotoxic factor in AHP ascitic fluid is active at very low concentrations.

Dose-response studies (see Fig. 3, 4) indicate that the increase in mitochondrial state 4 respiration is positively correlated to the concentration of canine or human PAAF used. This suggests that the organ damage observed in AHP may be proportional to the toxicity of the PAAF. Although the nature of the toxic factor(s) is still under investigation, it is evident from the work of Ellison *et al.* (7) and Pappas *et al.* (10) that it is not due to electrolyte imbalance, pH, amylase, or trypsin activity. Enzymes such as elastase and lecithinase, low molecular weight proteins, prostaglandins, and fatty acids cannot as yet be excluded as possible toxic factors. One possibility to explain the changes seen in AHP could be the presence of endotoxin. Our studies demonstrate that sterile PAAFs, showing a negative endotoxin assay, are active at high dilutions in damaging mitochondrial respiratory control.

The rat mitochondrial assay appears to be a sensitive method for evaluating the presence of toxic factors in PAAF. The canine PAAFs tested showed an average increase in state 4 respiration of 120% and all preparations failed to respond to readdition of ADP after PAAF exposure, indicating that mitochondrial uncoupling had occurred. We compared the mitochondrial bioassay with Ellison and coworkers' (7) hemoconcentration studies, and found that all the PAAFs they reported to cause hemoconcentration after ip injection in rats were able to uncouple oxidative phosphorylation of rat liver mitochondria. The strong correlation between our and Ellison's data suggests the multifaceted toxic activity of PAAF.

Assays currently used to test for PAAF toxicity, such as lethality in mice after ip injection (15), fall in blood pressure (6), respiratory failure (16), increase in hematrocrit (7), or isolated liver cell oxygen uptake (10), are not as sensitive as the mitochondrial technique.

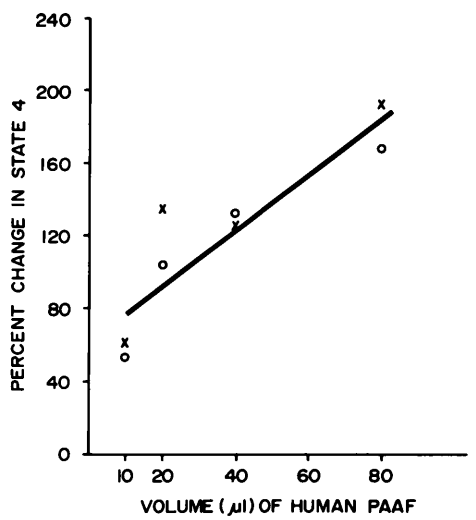

FIG. 3. Dose-response curve for two different human PAAFs. The solid line was generated by linear regression.

The mitochondrial test can be set up in less than 2 hr and requires very small amounts (μl) of either PAAF or PAAF fractions. It is a fairly rapid, highly reproducible technique that allows for replicate assays (we did four repeats for each PAAF) with the same or subsequent day mitochondrial preparations. The measurement of mitochondrial respiratory activity with very small amounts of PAAF stands in contrast to the other PAAF toxicity tests (except for liver cell oxygen uptake) which require relatively large volumes of PAAF or PAAF fractions, many test animals, and have a limited sensitivity because of the relatively small changes observed. The mitochondrial method was compared with the isolated liver cell technique described by Pappas *et al.* (10) and gave comparable results with lower concentrations of PAAF. Since both of these procedures were developed in our laboratory, we are well aware of the technical aspects of each test and feel that the mitochondrial assay is better suited for tests of PAAF fractions where only small subsamples of isolate may be available.

The mitochondrial respiratory assay technique was used in an ongoing double-blind study of canine PAAFs and PAAF fractions. These experiments indicate that the factor or factors that Ellison *et al.* (7) demonstrated to cause hemoconcentration on ip injection in rats, also cause hepatic mitochondrial uncoupling. Ellison's group, using the same PAAF we later tested in our double-blind study, found the toxic factor to be "stable to boiling for 10 min, stable to trichloracetic acid precipitation, resistant to ether extraction," and could be ultrafiltered through a 1000-dalton Amicon filter. Based on these data they suggest that the toxic factor is probably a small protein molecule.

Our current studies with trichloracetic acid precipitated PAAF samples, extracted with ether, lyophilized, and later redissolved in phosphate-buffered saline, indicate that the toxic factor does not appear to be in the aqueous layer and may reside in the residue of the ether layer. Free fatty acids (FFA) released in AHP can induce respiratory failure in the isolated dog lung (16) and cause uncoupling of mitochondrial oxidative phosphorylation (17) which results in a depletion of ATP in mitochondria. FFA, which have relatively low molecular weights, are released in AHP due to the lipolytic activity of pancreatic lipases (18). Pappas *et al.* (19) showed that PAAF has high concentrations of FFA, but argues that FFA should increase rather than decrease liver cell respiration. This is an effect opposite to that found in their isolated liver cell studies (10). Our work with hepatic mitochondria shows that active PAAF fractions significantly increased state 4 respiration, and we suggest that this will result in a depletion of ATP and a fall in endogenous respiratory activity of liver cells such as that observed by Pappas *et al.* (10).

With the use of the sensitive hepatic mitochondrial assay described in this paper, we are presently testing fractions of PAAF samples to more precisely identify the nature of the toxic factor(s) involved in AHP. These studies will aid in understanding the pathological mechanisms involved in AHP and hopefully will lead to improved therapeutic measures in the treatment of AHP.

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1. Carey LC. Extra-abdominal manifestations of acute pancreatitis. Surgery **86**:337–342, 1979.
2. Barbezat GD, Waterworh MW. Atrial fibrillation in acute pancreatitis. S Afr Med J **53**:554–555, 1978.
3. Butler ML. Abnormalities of liver function in acute pancreatitis. South Med J **66**:700–702, 1973.
4. Balsløv JT, Jørgensen HE, Nielsen R. Acute renal failure complicating severe acute pancreatitis. Acta Chir Scand **124**:348–354, 1962.
5. Ranson JHC, Turner JW, Rose DF, Rifkind KM, Spencer FC. Respiratory complications in acute pancreatitis. Ann Surg **179**:557–566, 1974.
6. Amundsen EE, Ofstad E, Hagen PO. Experimental acute pancreatitis in dogs. 1. Hypotensive effect induced by pancreatic exudate. Scand J Gastroenterol **3**:659–664, 1968.
7. Ellison EC, Pappas TN, Johnson JA, Favri PJ, Carey LC. Demonstration and characterization of the hemoconcentrative effect of ascitic fluid that accumulates during hemorrhagic pancreatitis. J Surg Res **30**:241–248, 1981.
8. Rodgers RE, Carey LC. Peritoneal lavage in experimental pancreatitis in dogs. Amer J Surg **111**:792–794, 1966.
9. Rosato EF, Mullis WF, Rosato FE. Peritoneal lavage therapy in hemorrhagic pancreatitis. Surgery **74**:106–115, 1973.
10. Pappas TN, Lessler MA, Ellison EC, Carey LC. Pan-

creatitis-associated ascitic fluid: Effect on the oxygen consumption of liver cells. Proc Soc Exp Biol Med **169**:438–444, 1982.
11. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation: III. The steady state. J Biol Chem **217**:409–428, 1955.
12. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem **177**:751–766, 1949.
13. Lessler MA, Scoles PV. Respiratory activity of isolated chondrocytes with a miniaturized oxygen electrode system. Ohio J Sci **80**:262–268, 1980.
14. Kitamura O, Ozawa K, Honjo I. Alterations of liver metabolism associated with experimental acute pancreatitis. Amer J Surg **126**:379–382, 1973.
15. Frey CF, Wong HN, Hickman D, Pullos T. Toxicity of hemorrhagic ascitic fluid associated with hemorrhagic pancreatitis. Arch Surg **117**:401–404, 1982.
16. Kimura T, Toung JK, Margolis S, Bell WR, Cameron JL. Respiratory failure in acute pancreatitis: The role of free fatty acids. Surgery **87**:509–513, 1980.
17. Borst P, Loos JA, Christ EJ, Slater EC. Uncoupling activity of long-chain fatty acids. Biochim Biophys Acta **62**:509–518, 1962.
18. Edmondson HA, Berne CJ. Calcium changes in acute pancreatic necrosis. Surg Gynecol Obstet **79**:240–244, 1944.
19. Pappas TN, Gavino VC, Ellison EC, Cornwell DG, Pace WG, Carey LC. Concentration of free fatty acids in pancreatitis-associated ascitic fluid. Clin Chem **27**:358, 1981.

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