The Cholesteryl Octanoate Breath Test: A New Procedure for Detection of Pancreatic Insufficiency in the Rat

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ABSTRACT. A breath test for the detection of pancreatic insufficiency was developed and tested in rats. The test features the hydrophobic molecule cholesteryl-1-¹⁴C-octanoate, which liberates ¹⁴C-octanoic acid when hydrolyzed by carboxyl ester lipase (cholesterol esterase). The ¹⁴C-octanoate is absorbed passively and rapidly metabolized to ¹⁴CO₂, which is excreted in expired air. The compound was administered as an emulsion of cholesteryl octanoate, triglyceride, and lecithin to rats with mild pancreatic insufficiency induced by injecting the pancreatic duct with zein. The animals had exocrine pancreatic hypofunction based on the enzyme content of pancreas at autopsy. Amylase was reduced by $97.1 \pm 1.4\%$, whereas chymotrypsin was reduced by $73 \pm 14\%$. The *p*-aminobenzoic acid test was abnormal at 1 wk ($21.68 \pm 8.4\%$), but become normal at 3 months ($72.08 \pm 5.8\%$) after zein injection. Despite this, the animals gained weight and absorbed fat normally. The ¹⁴CO₂ excretion rate in the 110-min interval after feeding was significantly reduced to 60% of sham-operated animals. Peak ¹⁴CO₂ collections 20 min after feeding were reduced by $75 \pm 11\%$. ¹⁴CO₂ output was completely normalized by administration of pancreatin prior to the test meal. The results suggest that a sensitive, noninvasive method for detecting deficiency of pancreatic carboxyl ester lipase (cholesterol esterase) secretion in the rat has been developed. (*Pediatr Res* 22: 257-261, 1987)

Abbreviations

NBT-PABA, N-benzoyl-L-tyrosyl-*p*-aminobenzoic acid
TLC, thin-layer chromatography

The diagnosis of pancreatic disease, particularly in cases of cystic fibrosis or other congenital abnormalities of the pancreas, remains a continuing problem. The conventional procedure to assess pancreatic function requires the measurement of total fatty acids in stool samples collected for 3 to 4 days. This procedure is not only inconvenient and cumbersome for the parents, it is also difficult to perform in infants who are not toilet trained. Furthermore, the presence of fat malabsorption is likely to be a rather late symptom, since steatorrhea does not occur in adults

until severe pancreatic insufficiency, manifested by a 90% reduction in enzyme secretion, is present (1). At this stage the diagnosis is usually obvious because of the presence of steatorrhea or other manifestations of the disease. Consequently, the diagnostic challenge is to detect pancreatic disease before severe malabsorption occurs.

It is agreed that the most reliable method of assessing pancreatic function involves duodenal intubation for direct measurement of pancreatic enzymes after stimulation with cholecystokinin-secretin, secretin, or Lundh meal. This approach is not only invasive, time consuming, and expensive, it is especially difficult to perform in children. Thus, many have attempted to develop a noninvasive, easy, and sensitive test to assess pancreatic function (2). For some years a number of investigators have been exploring the utility of breath tests, attracted by their simplicity and low cost (3). In 1962, Schwabe *et al.* (4) reported promising results with a ¹⁴CO₂ breath test using trioctanoin. A comprehensive study by Newcomer *et al.* (5) showed that a ¹⁴CO₂ test based on triolein gave better results. The triolein test was sensitive, but likely to give false-positive results in biliary and intestinal disease. In addition, the test required 4 to 6 h to perform and had considerable radiation hazard.

The present study was performed to develop a new breath test for measurement of intraluminal fat digestive activity and in particular to assess the feasibility of a ¹⁴CO₂ breath test based on the hydrolysis of cholesteryl-1-¹⁴C-octanoate as a new marker for exocrine pancreatic function.

METHODS

Principle of the test. A graphic illustration of the cholesteryl octanoate breath test is shown in Figure 1. The test molecule cholesteryl-1-¹⁴C-octanoate is a cholesterol ester with an 8-chain fatty acid in the ester position. After ingestion, the marker is hydrolyzed by pancreatic carboxyl ester lipase (cholesterol esterase) to cholesterol and ¹⁴C-octanoic acid. Octanoate is then passively and rapidly absorbed by the intestinal mucosa, transported without prior esterification via the portal vein to the liver and oxidized to ¹⁴CO₂. The rate at which ¹⁴CO₂ is expired, is assumed to be proportional to the rate of hydrolysis of the marker, *i.e.* in the multiple steps between ingestion of the compound and its recovery as ¹⁴CO₂ in breath, esterolysis is hypothesized to be rate limiting.

Male Sprague-Dawley rats weighing between 280 and 340 g were used. They were housed separately in regular cages with free access to water and a standard pellet diet.

Pancreatic exocrine hypofunction was induced according to Rhodes *et al.* (6). After an overnight fast, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (3 mg/100 g) and the abdomen was opened by a midline incision.

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Fig. 1. Principle of the cholesteryl-1- ^{14}C -octanoate breath test for the detection of pancreatic exocrine hypofunction.

The pancreas was identified and a silk suture was passed under the duct near the duodenum for stabilization and temporary occlusion. The biliary duct proximal to the junction of the biliary and pancreatic ducts was occluded temporarily with a forceps to prevent the zein solution from entering the liver. The zein solution (0.1 ml/100 g of rat) was injected into the distal duct through a 27-gauge needle attached to a syringe. After closing the abdomen, the animals were returned to their individual cages. Water and food were again offered *ad libitum*. Sham-operated rats served as control animals. The zein solution contained per ml 37 mg zein (Sigma, St. Louis, MO) dissolved in 0.1 ml of 95% ethanol and suspended in 0.9 ml of linoleic and oleic acids (2:1 v/v) (Sigma).

To confirm pancreatic hypofunction, a NBT-PABA test was performed 1 wk after the injection of the zein solution. The animals were fasted over night and 1 mg/100 g rat of Bentiromide (N-benzoyl-L-tyrosyl-paraaminobenzoate) in 2 ml solution was injected in the stomach through a blunt tipped metal cannula passed down the esophagus. Food and water were offered *ad libitum* after gastric injection and urine was collected for 24 h. Urine paraaminobenzoic acid was determined by the Bratton-Marshall reaction after acid hydrolysis (7).

Before breath testing, the animals were kept for 3 months in individual cages with free access to a standard pellet diet, to give time for possible intestinal adaptation to pancreatic hypofunction. After this time interval, the cholesteryl-1- ^{14}C -octanoate breath test was performed, and pancreatic function was determined by measurement of NBT-PABA excretion and fat absorption. The animals were then sacrificed, and the chymotrypsin and amylase content in total pancreatic homogenate was determined.

Considerable care was taken to develop a method of emulsifying the practically water-insoluble cholesteryl octanoate in such way that the emulsion was stable and the particle size was small. Preliminary experiments indicated that the nature of the emulsion markedly influenced $^{14}\text{CO}_2$ output. The optimal result, i.e. the greatest recovery of $^{14}\text{CO}_2$, was obtained by using 1:10:12 (w/w/w) proportions of cholesteryl octanoate, triglyceride, and lecithin. To prepare the emulsion, 5 mg cholesteryl octanoate, 50 mg triglyceride, and 60 mg lecithin were dissolved in chloroform in a 20-ml glass liquid scintillation vial. The chloroform was evaporated under a N_2 -stream so that the substrate remained as a thin layer adherent to the bottom of the vial. Two and one-half ml of NaCl 0.9% were added, and the solution was sonicated in a Brownwill Biosonic (Brownwill Scientific, Rochester, NY) for 20 min with an energy output of 200 W in an ice bath under an N_2 stream (test meal I). The test meal was freshly prepared for each set of experiments, using this technique. The radioactiv-

ity concentration of the emulsion was measured prior to administration of the test meal.

To exclude impaired absorption and/or metabolism of octanoate in zein-injected rats, a test meal containing the products of hydrolysis of cholesteryl octanoate (1.9 μmol cholesterol and 1.9 μmol ^{14}C -octanoic acid) was administered as a positive control (test meal II). Preparation of the test meal was identical to the one described above.

Another positive control was performed by feeding zein-injected rats with pancreatic enzymes 5 min prior to administration of test meal I. Pancreatic enzymes consisted of 1/4 capsule Cotazyme which was equal to 2000 USP units lipase, 7500 USP units protease, and 7500 units amylase. The capsule was suspended in 5 ml of isotonic saline (test meal III).

Animals were fasted over night prior to each experiment. After administration of 0.5 ml of the test meal, containing 1 mg of cholesteryl octanoate (200,000 dpm) by an intragastric tube, the animals were immediately placed in the glass cage of the CO_2 trapping train.

Test meal I (cholesteryl- ^{14}C -octanoate), test meal II (cholesterol and ^{14}C -octanoic acid), and test meal III (cholesteryl- ^{14}C -octanoate and pancreatin) were administered to zein-injected rats, whereas control animals received only test meals I and II.

The CO_2 trapping train consisted of a glass cage for the animal with a volume of 1.8 liter and four gas bubblers connected by tubing to a vacuum line. The trapping solution in the bubbler consisted of 5 ml ethanolamine and methoxyethanol (1:1 v/v). CO_2 trapping was highly efficient, leading to a 95% retention of radioactivity in the first bubbler and more than 4% in the second bubbler. Less than 1% of radioactivity was lost. Samples were counted after adding 4 ml of methanol and 10 ml of a Toluene-based scintillation cocktail (AquaSol NEN) to the trapping fluid. Counts were converted to disintegrations per min by external standardization. The results were expressed either as percentage of administered dose per min or as cumulative recovery in percent of administered dose.

For feces collection, animals were housed in individual metabolic cages with free access to water and a standard diet containing 6% fat. After 3 days, feces were collected and food intake was measured for 3 days. The 3-day stool was pooled, freeze dried, and investigated for fecal fat according to Jover and Gordon (8).

For *in vitro* amylase and chymotrypsin measurements the rats were sacrificed using ether and the pancreas was removed. The tissue was homogenized in 5 ml isotonic saline at 4°C . Amylase activity was estimated in the total pancreatic homogenate using the starch-iodine procedure (9), and chymotrypsin activity was measured with bentiromide after trypsin activation (10).

Cholesteryl-1- ^{14}C -octanoate was synthesized and supplied by Dr. Herok of Hoechst AG, Frankfurt/Main, West Germany. Radiopurity was checked by TLC, HPLC and GC and was found to be 99%. The specific activity was 22.4 mCi/g.

Nonradioactive cholesteryl octanoate was synthesized by reacting cholesterol with octanoyl chloride in water-free pyridine. The product was purified by several recrystallization steps in ethanol and its purity was checked by TLC. Lecithin was prepared from hen's egg yolk according to Singleton *et al.* (11). For preparation of the test meal, cholesterol-free vegetable oil was used. Cholesterol A grade was obtained from CalBioChem and ^{14}C -octanoic acid was purchased from NEN. Statistical analysis was conducted using the Wilcoxon rank sum test.

RESULTS

Figure 2 shows the $^{14}\text{CO}_2$ recovery over a 110-min period expressed as percent of dose administered after feeding 1 mg of ^{14}C -labeled cholesteryl octanoate (test meal I) to zein-injected rats and control animals. The appearance of $^{14}\text{CO}_2$ in the breath of control animals was very rapid, leading to a peak of radioactivity output as soon as 10–20 min after feeding. In contrast to these findings, $^{14}\text{CO}_2$ output in zein-injected rats increased slowly after feeding; in most rats peak collections were delayed

Fig. 2. $^{14}\text{CO}_2$ recovery rate (% dose/min) after administration of test meal I (cholesteryl-1- ^{14}C -octanoate) in zein-injected rats versus sham-operated control animals. * $P < 0.01$; mean $\pm$ SD.

until the fourth to sixth collection interval. At this time peak excretion was still less than half that of control animals. $^{14}\text{CO}_2$ excretion 20 min after feeding was significantly reduced to $25 \pm 11\%$ of control values in zein-injected animals.

Figure 3 shows the effect of feeding pancreatin prior to the test meal on the $^{14}\text{CO}_2$ recovery in zein-injected rats. Values obtained without pancreatin supplementation are also shown. The time course and rate of $^{14}\text{CO}_2$ output were restored fully to control values by enzyme supplementation.

The results of $^{14}\text{CO}_2$ excretion after feeding hydrolyzed substrate (test meal II) are shown in Figure 4. No difference was seen between controls and zein-injected animals, indicating that absorption and metabolism of octanoic acid was not impaired in animals with pancreatic insufficiency. Controls showed no significant difference between $^{14}\text{CO}_2$ recovery after test meal I, when compared to test meal II.

Cumulative recovery of radioactivity after 20 min or after 110 min is shown in Figure 5 after feeding different test meals to control animals and zein-injected rats. The overall recovery in zein-injected rats was reduced by 40% after administration of test meal I. Feeding test meal II (1.9 μmol cholesterol and 1.9 μmol ^{14}C -octanoic acid) fully restored $^{14}\text{CO}_2$ recovery in zein-injected rats to control values. In zein-injected rats $^{14}\text{CO}_2$ output was practically identical to control animals when pancreatic enzymes were administered prior to feeding cholesteryl octanoate (test meal III).

All experiments were performed in duplicate. There was an overall variation in total recovery within one animal of less than 4.6% and between different animals (controls) of less than 5.1%.

Table 1 summarizes the results of the fecal fat measurements and the NBT-PABA test. The fat absorption of zein-injected rats was $95.7 \pm 2.3\%$ and in the sham-operated animals it was $96.8 \pm 0.5\%$. The difference was not significant.

The results of the NBT-PABA test, expressed as percentage of dose recovered in 24 h, showed a significant difference between control animals and zein-injected rats 1 wk after injection of the zein solution. Three months after the operation, the results were similar in the two groups and no statistical significant difference was found. One wk after operation, zein-injected rats excreted $21.68 \pm 8.4\%$, whereas 3 months after operation PABA excretion was $72.08 \pm 5.8\%$.

The enzyme content of the total pancreatic homogenate showed a considerable degree of reduction in exocrine function (Table 2). Amylase was reduced by $97 \pm 1.4\%$, while chymotrypsin was reduced by $71 \pm 14\%$ in zein-injected rats when compared to control values.

DISCUSSION

Herein we describe a new breath test for the rapid and simple indirect assessment of pancreatic lipolytic activity in the rat. The

Fig. 3. Effect of prefeeding of pancreatin on $^{14}\text{CO}_2$ recovery rate (% dose/min) in zein-injected rats (test meal III). Data from animals not receiving pancreatin are also shown (test meal I). * $p < 0.01$; NS to control (Fig. 2); mean $\pm$ SD.

Fig. 4. $^{14}\text{CO}_2$ recovery rate (% dose/min) after administration of test meal II (cholesterol + ^{14}C -octanoate) in zein-injected rats versus sham-operated controls. NS to control; mean $\pm$ SD.

Fig. 5. Cumulative $^{14}\text{CO}_2$ recovery in 20 min (right) and 110 min (left) in zein-injected rats versus sham-operated control animals after administration of test meal I, test meal II (cholesterol + ^{14}C -octanoic acid), and test meal III (pancreatin prefed). * $p < 0.01$; mean $\pm$ SD.

test features the hydrophobic molecule cholesteryl- ^{14}C -octanoate, which liberates ^{14}C -octanoic acid, when hydrolyzed by pancreatic carboxyl ester lipase.

Cholesteryl octanoate appears attractive as a test molecule for

Table 1. Intake and excretion of fatty acids (FA) (mEq/day) and excretion of PABA (%dose/24 h) in zein-injected rats versus sham-operated control animals (mean $\pm$ SD)

	Zein (n = 5)	Control (n = 5)
FA (mEq/day)		
Intake*	5.48 $\pm$ 0.93	6.09 $\pm$ 1.1
Excretion*	0.24 $\pm$ 0.14	0.2 $\pm$ 0.04
Absorption (%)*	95.7 $\pm$ 2.3	96.8 $\pm$ 0.5
PABA excretion (%)		
1 wk after†	21.68 $\pm$ 8.4	65.0 $\pm$ 9.2‡
3 mo after zein injection*	72.08 $\pm$ 5.8	65.33 $\pm$ 28.5

* NS.

† $p < 0.01$.

‡ $n = 8$.

Table 2. Enzyme content in total pancreatic homogenate in zein-injected rats versus sham-operated control animals (mean $\pm$ SD)

	Zein (n = 5)	Control (n = 4)
Amylase*		
U/pancreas	1945 $\pm$ 950	66875 $\pm$ 17918
% control	2.9 $\pm$ 1.4	
Chymotrypsinogen*		
U/pancreas	3.2 $\pm$ 0.5	11.85 $\pm$ 3.37
% control	27 $\pm$ 14	

* $p < 0.01$.

pancreatic function for several reasons. In the intestinal lumen, cholesterol esters are hydrolyzed by carboxyl ester lipase (cholesterol esterase) to cholesterol and fatty acid (12), which subsequently can be absorbed through the mucosa (13). Enzymatic hydrolysis of dietary fat begins in the stomach, where 10–40% of the total dietary triglyceride may be digested (14). In humans and most mammals, the major gastric lipase is lingual lipase, an enzyme secreted from lingual serous glands (15). In contrast to triglycerides, phospholipids and cholesterol esters are resistant to lingual lipase (16). In addition to being present in pancreatic secretion, carboxyl ester lipase activity is also measurable in the intestinal mucosa. This enzymatic activity is believed to be of pancreatic origin, since rats subjected to external drainage of pancreatic juice show an absence of carboxyl ester lipase in the intestine (17). Hence, carboxyl ester lipase is a digestive enzyme secreted mainly, if not exclusively, by the exocrine pancreas. The value of cholesterol esterase as a marker enzyme for exocrine pancreatic insufficiency is supported by the encouraging results with the fluorescein dilaurate test (18), which also measures the activity of carboxyl ester lipase (cholesterol esterase) (19).

Peak recovery of $^{14}\text{CO}_2$ in breath occurred as early as 20 min after administration of the test meal. The rapid recovery of $^{14}\text{CO}_2$ is believed to be due to the unique properties of octanoic acid, namely its rapid, passive absorption (20) and the absence of esterification in the enterocyte, its transport to the liver in portal blood, and its rapid and complete oxidation in the liver or other tissues (21). In contrast to long-chain fatty acids, which are transported via the thoracic duct, most short-chain fatty acids, *i.e.* those with less than 12 carbon atoms, are absorbed directly into the portal venous system (22) and do not require bile acids for absorption, since they have considerable water solubility (23). However, pancreatic carboxyl ester lipase does require bile salts to be present at micellar concentration for full enzyme activity (12).

The functional degree of pancreatic insufficiency was determined by the result of fat absorption and the NBT-PABA test,

which was performed 1 wk after and 3 months after the injection of zein. Although the enzyme content in pancreatic homogenate revealed a significant degree of pancreatic insufficiency, fat and PABA excretion appeared to be normal at the time of breath testing. The observed shift in PABA excretion from significantly reduced levels right after the injection of zein to control values 3 months later, could be explained in a number of ways. A regeneration of destroyed pancreatic tissue seems unlikely in this model of pancreatic insufficiency (6). Although the NBT-PABA test has been successfully used to assess pancreatic function in humans (24) and rats (7), certain limitations regarding the tests specificity and sensitivity have been reported. PABA excretion was found after exclusion of pancreatic secretions from the intestine in rats (25) and after total pancreatectomy in humans (18). Certain nonpancreatic enzymes of bacterial and mucosal origin were shown to be capable of NBT-PABA hydrolysis (26, 25). It could be speculated that in long-standing pancreatic insufficiency bacterial overgrowth and/or adaptation of mucosal enzymes leads to recovery of NBT-PABA hydrolysis.

In zein-injected rats, the reduction of chymotrypsin activity in the pancreatic homogenate was much less pronounced than the reduction of amylase activity. Similar discordant changes of enzyme content were observed in the duct ligation model of pancreatic insufficiency, where amylase was found to be decreased by 54%, while trypsin was reduced by only 33% (27). In accordance to these findings, we reported in a recent study that the relative content of protease in pancreatic homogenate rises with the degree of pancreatic insufficiency (28). This shift in enzyme production seems to be an adaptive process of the remaining exocrine pancreas to the loss of exocrine tissue. The nonproportional increase of trypsin and chymotrypsin might be another explanation for the insensitivity of the PABA test in this situation.

Zein-injected rats did not show an impairment in fat absorption. Similar observations were made with another model for pancreatic insufficiency. Again, rats with severe pancreatic insufficiency had a normal fat absorption, when fed a standard diet (Mundlos S, Adler G, Hausmann W, unpublished observations) despite a 99% reduction of lipase activity in pancreatic homogenate. This somewhat surprising capacity of fat digestion in severe pancreatic malfunction might be due to adaptive processes leading to significant lipase production from sites other than the pancreas. In the rat, as well as in humans, lingual lipase and gastric lipase are known to play an important role in fat digestion (15). Initial lipolysis in the stomach by lingual and/or gastric lipase becomes a much more important process in conditions of pancreatic insufficiency and may in part compensate the lack of pancreatic digestive capacity (29). It was suggested that predoduodenal, *i.e.* nonpancreatic digestion, contributes to the assimilation of 60–70% of ingested fat in children with cystic fibrosis or congenital lipase deficiency (30). Similar adaptive processes in the rat seem likely.

Since the time course of $^{14}\text{CO}_2$ excretion after cholesteryl-14C-octanoate was practically identical to that after cholesterol and 14C-octanoic acid, it seems unlikely that lipolysis was the rate-limiting step in the process involving digestion, absorption, and metabolism of cholesteryl octanoate in control animals. It is assumed that in control animals the metabolism of the octanoic acid to $^{14}\text{CO}_2$, rather than hydrolysis, was the important determinant of $^{14}\text{CO}_2$ excretion. However, this was not the case in zein-injected rats, as either feeding of pancreatic enzymes prior to the test meal or administration of hydrolyzed cholesteryl-14C-octanoate fully restored the depressed $^{14}\text{CO}_2$ excretion. Thus, the rate-limiting step was lipolytic digestion, *i.e.* hydrolysis of the marker molecule, due to the lack of pancreatic enzymes.

Our data suggest that cholesteryl-14C-octanoate can be used as a noninvasive procedure to assess exocrine pancreatic function in experimental animals. The procedure described herein has been modified and in preliminary studies gives similar results in adults with pancreatic exocrine insufficiency (31). In this study,

cholesteryl-13C-octanoate was used successfully, suggesting that the test can be used as a test for pancreatic function in children. A prospective comparison of this new method with other non-invasive tests such as the PABA or the pancreolauryl test for sensitivity and specificity appears to be indicated.

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