

Measurement of expired carbon dioxide to assess the metabolism of remnant lipoproteins

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Abstract The cholesteryl esters transported from the intestine in chylomicrons are delivered to the liver. Hepatocytes take up chylomicron remnants by receptor-mediated endocytosis and the cholesteryl esters are subsequently degraded. In this study we measured the appearance in breath of labeled carbon dioxide after injection of chylomicron-like emulsions labeled with radioactive cholesteryl [1-¹⁴C]oleate. Measurements by the breath test provide an integrated assessment of capacity for clearance and subsequent metabolism of the remnants of the triglyceride-rich lipoproteins. In normal rats, mice, and rabbits injected with the radioactive emulsions, label appeared in the breath after a delay of approximately 30 min, appreciably slower than the appearance of label after injection of emulsions labeled with [¹⁴C]triolein or of [¹⁴C]oleic acid complexed with albumin. To test for the ability of the procedure to detect defects in remnant clearance, labeled emulsions were injected into diabetic rats, apoE-deficient mice, low density lipoprotein receptor (LDLr)-deficient mice, and Watanabe heritable hyperlipidemic (WHHL) rabbits with defective low density lipoprotein receptors. In rats made diabetic by treatment with streptozotocin the appearance of ¹⁴CO₂ in breath was slower than in normal control rats. This finding was consistent with previous evidence from our laboratory that remnant clearance is defective in diabetic rats. In LDLr-deficient mice the appearance of ¹⁴CO₂ was slower when compared with control mice and in apoE-deficient mice the appearance of ¹⁴CO₂ was extremely small. In homozygous WHHL rabbits, the appearance of ¹⁴CO₂ in breath was much slower than in normal control rabbits, while in heterozygous WHHL rabbits an intermediate level of appearance was found, consistent with our previous findings of defective remnant clearance in WHHL rabbits. Emulsions with cholesterol omitted, previously found to be cleared from plasma much slower than chylomicron-like emulsions, had much slower appearance of label in breath. ■ The breath test as described is consistent with predicted metabolism of chylomicron remnants and therefore provides a useful means of assessment of remnant catabolism in the intact animal.—Redgrave, T. G., I. J. Martins, and B-C. Mortimer. Measurement of expired carbon dioxide to assess the metabolism of remnant lipoproteins. *J. Lipid Res.* 1995. 36: 2670-2675.

Supplementary key words remnants • triglyceride-rich lipoproteins • chylomicron-like emulsions • breath test • cholesteryl esters • cholesterol • diabetes mellitus • WHHL rabbits

Remnant lipoprotein particles are derived from triglyceride-rich lipoproteins of intestinal and hepatic origin after hydrolysis of most particle triglycerides by the enzyme lipoprotein lipase (1). The remnants transport cholesterol absorbed from the intestine and are normally rapidly removed from the blood plasma by receptor-mediated endocytosis, primarily into the liver. After endocytosis, the lipoproteins are transported into endosomes and eventually into lysosomes, where the lipid components are hydrolyzed and fatty acids become available for oxidative metabolism, in particular metabolism to carbon dioxide. For the present studies in rats, mice, and rabbits, a single injection into a vein was made of a microemulsion prepared to mimic the composition and physiology of triglyceride-rich lipoproteins. The microemulsion incorporated a tracer amount of ¹⁴C-labeled cholesteryl oleate, with the label in the oleate moiety for production of ¹⁴CO₂ by oxidative metabolism of the fatty acid produced from hydrolysis of the emulsion cholesteryl ester. Expired air was sampled at intervals over several hours after injection. Expired air was collected into vessels containing solutions to trap the CO₂ produced as a result of the recipient animal's metabolism. A large proportion of the injected isotope was expired within a few hours. The procedure yields novel and precise quantitative information on the recipient animal's capacity for metabolic handling of chylomicron remnants.

Abbreviations: CO, cholesteryl oleate; TO, triolein; LDLr, low density lipoprotein receptor; WHHL, Watanabe Heritable Hyperlipidemic rabbits; apoE, apolipoprotein E.

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METHODS

Preparation of emulsion

Emulsions were prepared by sonication and purified by density gradient centrifugation as previously described (2). The injected emulsions were prepared from mixtures of triolein, egg lecithin, cholesteryl esters, and cholesterol. To label the cholesteryl oleate or triolein constituents respectively, the mixtures also contained trace amounts of cholesteryl [1-¹⁴C]oleate or tri-[1-¹⁴C]oleoylglycerol (Amersham International Plc, Buckinghamshire, England). As described in detail previously (3), the mixtures were emulsified by sonication, and emulsion particles of average size 150 nm were separated from the crude sonicated emulsion by centrifugation in density gradients. The emulsion preparation was designed to produce particles of average size 150 nm because particle size affects the kinetics of clearance (4–7). Cholesterol was present in appropriate amounts for the injected emulsion to mimic the metabolism of the natural exogenous lipid transport particles (8). Without cholesterol, the remnants remain in the plasma for much longer than occurs physiologically with natural chylomicrons, the lipoproteins that transport fat from the intestine. Cholesteryl esters are present in natural chylomicrons and are an essential ingredient of the emulsion, required as a carrier for the isotopic label to ensure proper physical distribution of the label between the other constituents of the lipid emulsion particles. The composition of the injected microemulsion was (% by weight, n = 5) TO, 83.4 ± 0.40; CO, 4.1 ± 0.46; cholesterol, 1.4 ± 0.04, and egg phosphatidylcholine, 11.1 ± 0.46.

Animals

Normal Wistar rats of body weight approximately 200 g were obtained from the Animal Resources Centre, Murdoch, Western Australia. Diabetes was induced in one group of rats by injection of streptozotocin at a dose of 50 mg/kg into a tail vein. In these studies, 3 weeks after the treatment with streptozotocin, rats were shown to be diabetic by the presence of glucose in their urine. We previously reported that 3 weeks after the treatment with streptozotocin the concentration of blood glucose was increased and glucosuria was present, but plasma lipids were only slightly increased (2, 9, 10). Colonies of apoE knockout mice and LDLr knockout mice were established from progenitor stocks obtained from the Jackson Laboratories, Bar Harbour, ME. The mice were bred by sibling matings to obtain animals homozygous for the null mutation. C57BL/6J mice were used as controls. WHHL rabbits were from a colony maintained in the Biological Sciences Animal Unit at the University of Western Australia, derived from stock obtained from

Professor Watanabe. Heterozygous WHHL were obtained by cross-breeding half-lop rabbits with homozygous WHHL, and animals from the half-lop strain were used as controls. All procedures were subject to inspection and approval by the Animal Welfare Committee of the University of Western Australia.

Collection of expired carbon dioxide

Emulsions were injected intravenously and the amounts of radioactive CO₂ in the expired breath were measured over several hours subsequently. Recipient rats were prepared under barbiturate anesthesia with a cannula in a jugular vein for making intravenous injections. After awakening from the anesthetic, the animal was placed in a closed chamber through which a stream of room air was passed. Conscious mice were injected with emulsion via a tail vein and then placed in the closed chamber. Rabbits were placed in a closed chamber for collection of expired breath after intravenous injection of labeled emulsion into an ear vein. Experiments were commenced by injection of labeled emulsion intravenously, and the CO₂ in the air leaving the chamber was passed through solutions containing 0.21 mol/L phenylethylamine (Aldrich, Milwaukee, WI), 50 ml of Permafluor (Packard, Downers Grove, IL), 270 ml of methanol, and 410 ml of toluene (11). Aliquots of the solutions were added to scintillation fluid then counted by liquid scintillation spectrometry.

RESULTS

As shown in Fig. 1, label in emulsion triolein (the TO, triglyceride portion of the emulsion) appeared rapidly in the expired breath after injection in normal rats. With label in the cholesteryl oleate (CO) portion of the emulsion the appearance of radioactivity in the expired breath was delayed compared with triolein label, but by 90 min a comparable amount of the radioactivity had been expired. To confirm the essential requirement of cholesterol, the experiment was repeated with cholesteryl oleate label in an emulsion without cholesterol. By 90 min after injection, much less of the emulsion radioactivity had appeared in expired breath, in contrast to the emulsion containing cholesterol. This result was consistent with the slow plasma removal of remnants derived from emulsions lacking cholesterol as previously described (8).

To investigate the efficiency of the breath test in detecting changes in remnant clearance secondary to diabetes, labeled emulsions were injected into diabetic rats 3 weeks after they were treated with streptozotocin at a dose of 50 mg/kg (intravenously). Rats made diabetic in this way have defective clearance of chylomicron remnants from plasma (2, 10). Figure 2 shows that the

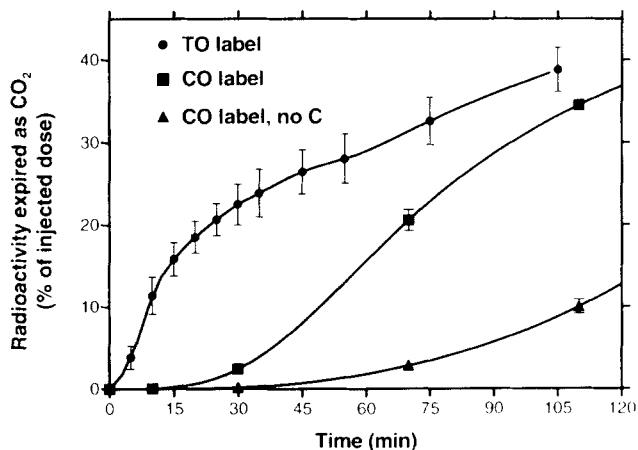


Fig. 1. The appearance of labeled CO_2 in expired breath after intravenous injection of emulsions labeled with cholesteryl [1- ^{14}C]oleate (CO) or glyceryl tri-[1- ^{14}C]oleoylglycerol (TO). Label in emulsion triolein appeared rapidly in the expired breath, with approximately 20% expired in 30 min. With label in the cholesteryl oleate portion of the emulsion only, a few percent of radioactivity appeared in the expired breath in 30 min, but by 90 min approximately 30% of the radioactivity had been expired. When the experiment was repeated with cholesteryl oleate label in an emulsion without cholesterol (no C), by 90 min after injection less than 10% of the emulsion radioactivity had appeared in expired breath, in contrast to the standard emulsion containing cholesterol. The results are means \pm SEM from 3 rats in each group.

expired radioactivity was decreased in the diabetic rats compared with non-diabetic normal control rats, illustrating the ability of the breath test to detect a defect in the ability to metabolize chylomicron remnants.

To check the specificity of the decrease in expired CO_2 from emulsions labeled in the CO moiety, control

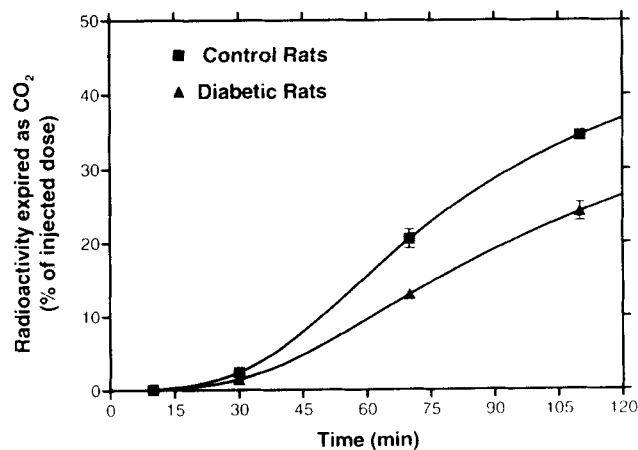


Fig. 2. The appearance of labeled CO_2 in expired breath after intravenous injection of emulsions labeled with cholesteryl [1- ^{14}C]oleate. The graph shows that the rate of expiration of the radioactive CO_2 was slower in diabetic rats than in normal control rats. Compared with approximately 30% expiration of the label in normal rats, diabetic rats expired only about 20% of the injected dose after 90 min. The results are means \pm SEM from 3 rats in each group.

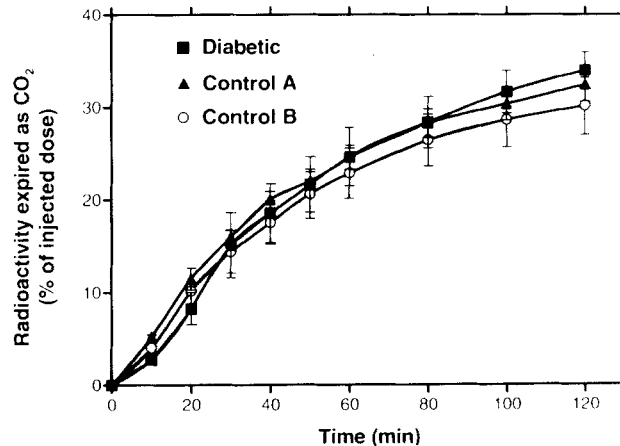


Fig. 3. The recovery of $^{14}\text{CO}_2$ in expired breath after injection of [^{14}C]TO-labeled emulsion in diabetic rats compared with non-diabetic control rats matched for age or for body weight. No differences in the appearance of $^{14}\text{CO}_2$ in the breath were found between either control or diabetic rats. Control A (matched for body weight), Control B (matched for age). The results are means \pm SEM from 3 rats in each group.

measurements were made in diabetic rats injected either with emulsion labeled with [^{14}C]TO or with [^{14}C]oleic acid complexed with albumin. **Figure 3** compares the recovery of $^{14}\text{CO}_2$ after injection of [^{14}C]TO-labeled emulsion in diabetic rats with non-diabetic control rats matched for age or for body weight (as diabetic rats gain weight at a slower rate than non-diabetic rats). It is clear that the appearance of $^{14}\text{CO}_2$ in breath was not decreased in the diabetic rats, in contrast to the decrease found when the label was in the CO moiety (Fig. 2). **Figure 4** shows that the appearance of $^{14}\text{CO}_2$ was not decreased when rats were injected with labeled oleic acid complexed with labeled albumin. The delay in appearance of label observed with injected emulsion containing [^{14}C]CO label (Fig. 2), therefore was not found with either [^{14}C]TO or [^{14}C]oleic acid labels.

Figure 5 compares the appearance of $^{14}\text{CO}_2$ in the expired breath of the control, LDLr-deficient, and apoE-deficient mice. The rate of expiration of $^{14}\text{CO}_2$ was much slower in the apoE-deficient mice than in the controls ($P < 0.0001$) by repeated measures of analysis. The radioactivity in expired breath was also significantly less in the LDLr-deficient mice compared with controls ($P < 0.0001$), indicating a defect or delay in the catabolism of remnants removed from the plasma.

As shown in **Fig. 6**, when labeled emulsions were injected intravenously, the rate of appearance of CO_2 in breath was slower in WHHL than in control rabbits ($P < 0.001$) by repeated measures of analysis of variance. In heterozygous WHHL rabbits, the rate of appearance of expired CO_2 was intermediate between control rabbits and homozygous WHHL rabbits ($P < 0.001$). This result was consistent with our previous findings of defective

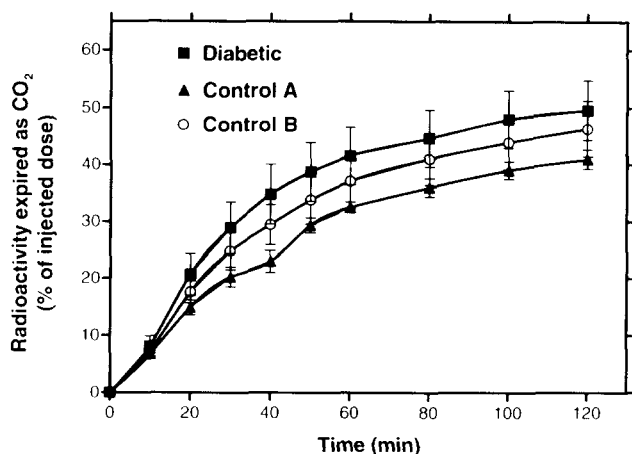


Fig. 4. This figure shows the appearance in expired breath of $^{14}\text{CO}_2$ after injection of [^{14}C]oleic acid complexed with albumin in diabetic rats compared with non-diabetic control rats matched for age or for body weight. The appearance of $^{14}\text{CO}_2$ in the breath was similar in control and diabetic rats; the small differences between the curves were not statistically significant by repeated measures of analysis of variance or by one-way analysis of variance at each time point. Control A (matched for body weight), Control B (matched for age). The results are means \pm SEM from 3 rats in each group.

plasma clearance of injected chylomicrons and chylomicron-like emulsions in WHHL (12, 13). In WHHL rabbits, LDL receptors are defective. Consequently, the clearance of LDL is slow and the concentration of LDL in plasma is increased, leading to the development of arteriosclerosis. Furthermore, the clearance of remnants of the triglyceride-rich lipoproteins is defective in WHHL, a reflection of the role of LDL receptors acting

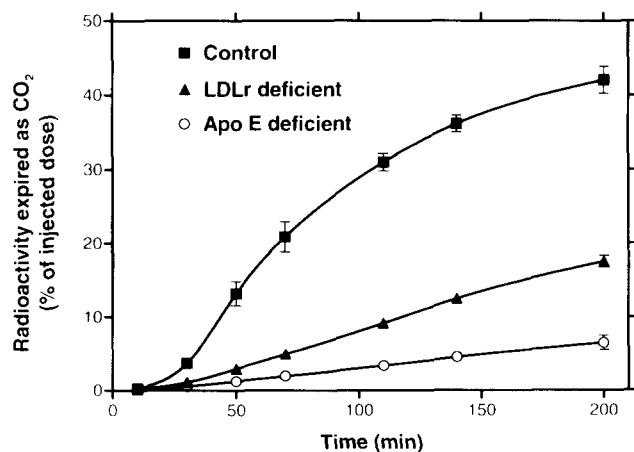


Fig. 5. The appearance of $^{14}\text{CO}_2$ in the expired breath of control C57BL/6, LDLr-deficient, and apoE-deficient mice. Mice were injected with 50 μl chylomicron-like emulsion labeled with cholesteryl [^{14}C]oleate via tail vein and radioactive $^{14}\text{CO}_2$ was collected as described in Methods. The rate of expiration of $^{14}\text{CO}_2$ was much slower in the apoE-deficient mice than in the control mice. The radioactivity in expired breath was also significantly less in the LDLr-deficient mice compared with controls. The results are means \pm SEM from 3 mice in each group.

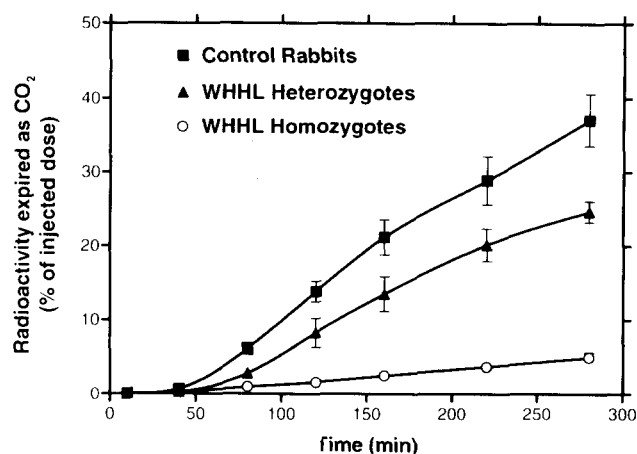


Fig. 6. The radioactivity in expired breath in homozygous and heterozygous WHHL rabbits compared with normal control rabbits. It is apparent from the graph that the rate of expiration of the radioactive CO_2 was much slower in the homozygous WHHL rabbits than in control rabbits. In heterozygous WHHL animals, the appearance of radioactivity in expired breath was intermediate. The results are means \pm SEM from 4 rabbits in each group for control and homozygote rabbits, and means \pm range from 2 rabbits in the heterozygote group.

as a ligand for the apolipoprotein E associated with remnants.

DISCUSSION

In his landmark review of cholesteryl ester metabolism, Goodman in 1965 (14) described how approximately 90% of cholesteryl ester was rapidly taken up by the liver of rats injected with labeled chylomicrons. Hydrolysis of the chylomicron cholesteryl esters occurred after a delay, and 3.5 h after injection hydrolysis was 80% complete. In 1965 the role of chylomicron remnants in the delivery of chylomicron cholesteryl esters to the liver had not been identified. It is nevertheless evident that while the underlying metabolic events have been known for 30 years, there is no facile procedure for measuring the catabolism of chylomicron cholesteryl esters in intact animals. The present work describes how the catabolism of chylomicron cholesteryl esters can be measured with minimal disturbance of the intact animal. The breath test described here provides an integrated assessment of remnant clearance and is simple to do. The method lends itself to repeated measurements to assess the effects of manipulations including pharmacological and nutritional interventions.

Breath tests are currently used in various applications, for example in the diagnosis of specific infections (15, 16), in assessment of gastric emptying (17), and in assessment of the activities of liver enzymes (18). Methods of testing of intestinal function using stable isotopes rely also on the same general principle of collection of

expired CO₂ (17, 19, 20) and many applications using this technology have been reported (16, 17, 21, 22). To avoid the use of a radioactive label, a stable isotope can be used followed by mass spectrometric quantitation of ¹³C₂O₂ in expired breath (19, 20, 23–28). We have made measurements of chylomicron remnant metabolism using [¹³C]cholesteryl ester labeled in the fatty acid moiety (T. G. Redgrave and I. J. Martins, unpublished observations). However, without using extensively labeled fatty acids, the isotope enrichment ratios were too low, and the amounts of emulsion required were too large to make the use of stable isotopes practical or cost-effective in this application.

The present studies in animals show that remnant clearance can be assessed simply and with minimal intervention by a breath test. The appearance of label in expired breath was impaired in several animal models where the plasma clearance of remnants is known by other methods to be defective. Control studies with labeled triolein and oleic acid have eliminated complications of interpretation due to other abnormalities of lipid metabolism in insulin-deficient rats.

The capacity to metabolize remnant lipoprotein particles contributes to the risk of atherosclerosis in man, as measured by the progression of coronary atherosclerosis determined angiographically (29, 30). Many previous studies point to an association between the abnormal metabolism of remnants of triglyceride-rich lipoproteins and the development of atherosclerosis. However, limitations in presently available methods have prevented a clear understanding of the possible contribution of remnant metabolism to arterial disease. Application of the breath test could bring a clearer understanding of this problem. ■

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REFERENCES

- Redgrave, T. G. 1983. Formation and metabolism of chylomicrons. *Int. Rev. Physiol.* **28**: 103–130.
- Redgrave, T. G., and M. J. Callow. 1990. The effect of insulin deficiency on the metabolism of lipid emulsion models of triacylglycerol-rich lipoproteins in rats. *Metabolism.* **39**: 1–10.
- Redgrave, T. G., H. L. Ly, E. C. Quintao, C. F. Ramberg, and R. C. Boston. 1993. Clearance from plasma of triacylglycerol and cholesteryl ester after intravenous injection of chylomicron-like lipid emulsions in rats and man. *Biochem. J.* **290**: 843–847.
- Lutz, O., Z. Meraihi, J. L. Mura, A. Frey, G. H. Riess, and A. C. Bach. 1989. Fat emulsion particle size: influence on the clearance rate and the tissue lipolytic activity. *Am. J. Clin. Nutr.* **50**: 1370–1381.
- Guldur, T., and P. A. Mayes. 1992. Metabolic comparison of small chylomicrons (intestinal VLDL) and large chylomicrons. *Biochem. Soc. Trans.* **20**: 104S.
- Takino, T., K. Konishi, Y. Takakura, and M. Hashida. 1994. Long circulating emulsion carrier systems for highly lipophilic drugs. *Biol. Pharm. Bull.* **17**: 121–125.
- Redgrave, T. G., J. C. L. Mamo, A. Bowler, and V. S. Mackintosh. 1992. Factors regulating hepatic uptake of remnant lipoproteins. In *Hepatic Endocytosis of Lipids and Proteins*. E. Windler and H. Greten, editors. H. W. Zuckschwerdt Verlag, Munich. 320–325.
- Redgrave, T. G., G. G. Vassiliou, and M. J. Callow. 1987. Cholesterol is necessary for triacylglycerol-phospholipid emulsions to mimic the metabolism of lipoproteins. *Biochim. Biophys. Acta.* **921**: 154–157.
- Callow, M. J., and T. G. Redgrave. 1993. Isoforms of rat apolipoproteins and changes induced by insulin deficiency and fasting. *Biochim. Biophys. Acta.* **1168**: 271–279.
- Mamo, J. C., T. Hirano, A. Sainsbury, A. K. Fitzgerald, and T. G. Redgrave. 1992. Hypertriglyceridemia is exacerbated by slow lipolysis of triacylglycerol-rich lipoproteins in fed but not fasted streptozotocin diabetic rats. *Biochim. Biophys. Acta.* **1128**: 132–138.
- Watkins, P. B. 1991. Breath tests as noninvasive assays of P450s. *Methods Enzymol.* **206**: 517–522.
- Bowler, A., T. G. Redgrave, and J. Mamo. 1991. Chylomicron-remnant clearance in homozygote and heterozygote Watanabe-heritable-hyperlipidaemic rabbits is defective - lack of evidence for an independent chylomicron-remnant receptor. *Biochem. J.* **276**: 381–386.
- Mamo, J. C. L., A. Bowler, C. A. Elsegood, and T. G. Redgrave. 1991. Defective plasma clearance of chylomicron-like lipid emulsions in Watanabe heritable hyperlipidemic rabbits. *Biochim. Biophys. Acta.* **1081**: 241–245.
- Goodman, D. S. 1965. Cholesterol ester metabolism. *Physiol. Rev.* **45**: 747–839.
- Marshall, B. J., and I. Surveyor. 1988. Carbon-14 urea breath test for the diagnosis of *Campylobacter pylori*-associated gastritis. *J. Nuclear Med.* **29**: 11–16.
- Marshall, B. J., M. W. Plankey, S. R. Hoffman, C. L. Boyd, K. R. Dye, H. F. Frierson, Jr., R. L. Guerrant, and R. W. McCallum. 1991. A 20-minute breath test for *Helicobacter pylori*. *Am. J. Gastroenterol.* **86**: 438–445.
- Ghoos, Y. F., B. D. Maes, B. J. Geypens, G. Mys, M. I. Hiele, P. J. Rutgeerts, and G. van Trappen. 1993. Measurement of gastric emptying rate of solids by means of a carbon-labeled octanoic acid breath test. *Gastroenterology.* **104**: 1640–1647.
- Watkins, P. B., D. K. Turgeon, P. Saenger, K. S. Lown, J. C. Kolars, T. Hamilton, K. Fishman, P. S. Guzelian, and J. J. Voorhees. 1992. Comparison of urinary 6-beta-cortisol and the erythromycin breath test as measures of hepatic P450III_A (CYP3A) activity. *Clin. Pharmacol. Ther.* **52**: 265–273.
- McClellan, P., M. Harding, W. A. Coward, M. R. Green, and L. T. Weaver. 1993. Measurement of fat digestion in early life using a stable isotope breath test. *Arch. Dis. Child.* **69**: 366–370.
- Hiele, M., Y. Ghoos, P. Rutgeerts, G. Vantrappen, and K. de Buyser. 1990. ¹³C₂O₂ breath test to measure the hydrolysis of various starch formulations in healthy subjects. *Gut.* **31**: 175–178.

21. Young, E. A., M. M. Harris, T. L. Cantu, S. M. Deneke, and S. Schenker. 1993. Use of the [^{14}C]aminopyrine breath test to assess the hepatic response of dietary obese rats to a very-low-energy diet. *Am. J. Clin. Nutr.* **57**: 863–867.
22. Kruger, N., H. Helge, and D. Neubert. 1991. CO_2 breath tests using ^{14}C -caffeine, ^{14}C -methacetin and ^{14}C -phenacetin for assessing postnatal development of monoxygenase activities in rats and marmosets. *Dev. Pharmacol. Ther.* **16**: 164–175.
23. Zanconato, S., D. M. Cooper, T. J. Barstow, and E. Landaw. 1992. $^{13}\text{CO}_2$ washout dynamics during intermittent exercise in children and adults. *J. Appl. Physiol.* **73**: 2476–2482.
24. Hiele, M., Y. Ghos, P. Rutgeerts, and G. Vantrappen. 1989. Starch digestion in normal subjects and patients with pancreatic disease, using a $^{13}\text{CO}_2$ breath test. *Gastroenterology*. **96**: 503–509.
25. Hiele, M., Y. Ghos, P. Rutgeerts, and G. Vantrappen. 1988. Measurement of the rate of assimilation of oligo- and polysaccharides by $^{13}\text{CO}_2$ breath tests and isotope ratio mass spectrometry. *Biomed. Environ. Mass. Spectrom.* **16**: 133–135.
26. Hiele, M., Y. Ghos, P. Rutgeerts, G. Vantrappen, H. Carchon, and E. Eggermont. 1988. $^{13}\text{CO}_2$ breath test using naturally ^{13}C -enriched lactose for detection of lactase deficiency in patients with gastrointestinal symptoms. *J. Lab. Clin. Med.* **112**: 193–200.
27. Ghos, Y., P. Rutgeerts, G. Vantrappen, and M. Hiele. 1988. Measurement of ^{13}C -glucose oxidation rate using mass spectrometric determination of the CO_2 :argon ratio and spirometry. *Biomed. Environ. Mass. Spectrom.* **15**: 447–451.
28. Metges, C. C., and G. Wolfram. 1993. Different (CO_2)-C-13 recovery of orally administered [1-C-13]- and [8-C-13]triolein in postprandial humans—an effect of phosphoenolpyruvate-carboxykinase (EC 4.1.1.32) in peripheral tissues. *Clin. Nutr.* **12**: 337–343.
29. Steiner, G. 1993. Triglyceride-rich lipoproteins and atherosclerosis, from fast to feast. *Ann. Med.* **25**: 431–435.
30. Phillips, N. R., D. Waters, and R. J. Havel. 1993. Plasma lipoproteins and progression of coronary artery disease evaluated by angiography and clinical events. *Circulation*. **88**: 2762–2770.