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Original article

Critical analysis of the use of ^{14}C-acetate for measuring *in vivo* rat cholesterol synthesis

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Summary — The bulk of cholesterol produced by the liver and the gut enters the mobile pool of body cholesterol. This process is called internal secretion in contrast with the fraction of biosynthesized cholesterol directly eliminated in the feces (fecal external secretion). In rats, under various conditions, a linear relationship was found between the rates of internal secretion measured by the isotope equilibrium method (range: 10–60 mg/day) and the sum of sterol radioactivities measured in liver and intestine 70 min after a [^{14}C]-acetate pulse. In fact, a better correlation was found between the radioactivities of liver sterols and the values for internal secretion. In this new relationship, the ordinate at the origin corresponds to a minimal internal secretion of about 10 mg/day, which implies an important extrahepatic cholesterol production, probably from the gut. Indeed, in adult male rats, fed a semi-purified sucrose-rich diet, the relative contribution of this organ to the internal secretion was higher than in adult rats fed a commercial diet and higher than in young animals, whatever the circadian period. It can be concluded that some of the discrepancies observed in the literature about the relative participation of the intestine and the liver in the internal secretion of cholesterol are probably due to differences in experimental and nutritional conditions (age and sex of the animals, diet composition, time of the circadian cycle) rather than to the cholesterol precursor used (^3H_2O or [^{14}C] acetate) to assess the activity of cholesterol synthesis. Indeed, a comparative study of ^3H_2O and [^{14}C]acetate incorporation into sterols of enterocytes indicated the same crypt-villus radioactive gradient, regardless of the intestinal site studied (duodenum, jejunum or ileum) and thus validated the use of [^{14}C]acetate. Other experiments however, showed evidence of some local differences in the cytosolic dilution of labeled acetyl CoA by the endogenous cholesterol precursor in rats under various conditions (control or cholestyramine-enriched diet, parenteral nutrition). After intravenous infusion of 1,2[^{13}C]acetate, mass fragmentography of free cholesterol isolated from liver and intestine indicated different ^{13}C-labeling patterns of newly synthesized molecules. They indicate that cholesterol is generally synthesized from acetyl CoA with a lower ^{13}C-content in the liver than in the intestine. The local endogenous flow of acetyl CoA used for cholesterol synthesis was about 2-fold higher in the hepatocytes than in the enterocytes. This conclusion was confirmed by the results obtained with several experimental groups exhibiting a large range of both internal secretion of cholesterol and sterol radioactivities in liver and intestine after [^{14}C]acetate injection. It was possible to calculate that the same sterol radioactivity in liver and intestine after [^{14}C]acetate injection corresponds to an internal cholesterol secretion, 1.9-fold higher in the liver than in the intestine. Numerous data presented in this paper argue in favor of the value of assessing local cholesterol synthesis in the rat on the basis of the sterol radioactivity after [^{14}C]acetate injection. However, because of some differences in the specific radioactivity of acetyl CoA used for cholesterol synthesis in the liver and intestine, it re-

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mains important to confirm (by using [^{13}C]acetate, for example) that these differences are relatively constant, regardless of the experimental conditions.

^3H_2O / isotope equilibrium method / internal secretion / ^{13}C-acetate / ^{13}C-enrichment / cytosolic dilution / acetyl CoA

Résumé — Analyse critique de l'utilisation de l'acétate ^{14}C pour la mesure *in vivo* de la synthèse du cholestérol chez le rat. *Une grande part du cholestérol synthétisé par le foie et l'intestin se déverse dans le pool de cholestérol mobile de l'organisme. Ce processus est appelé «sécrétion interne» par opposition à la «sécrétion externe fécale» qui concerne la fraction du cholestérol synthétisé par l'intestin et directement éliminé dans les fèces. Chez les rats soumis à des conditions physiologiques et alimentaires très variées, une relation linéaire est obtenue entre la vitesse de la sécrétion interne (10 à 60 mg/j) mesurée par la méthode d'équilibre isotopique et la somme des radioactivités des stérols dans le foie et l'intestin, 70 min après l'administration d'acétate-^{14}C. En fait, une meilleure corrélation est trouvée entre la radioactivité des stérols hépatiques et la sécrétion interne. Pour cette dernière, l'ordonnée à l'origine correspond à une sécrétion interne minimale de 10 mg/j ce qui suggère l'existence d'une cholestérogenèse extrahépatique importante d'origine probablement intestinale. De plus, chez le rat mâle adulte recevant une alimentation semi-synthétique riche en saccharose, la contribution relative de l'intestin à la sécrétion interne est plus élévée que chez le rat mâle adulte nourri d'une alimentation commerciale et plus forte également que chez le jeune rat quelle que soit la période du nycthémère. Plusieurs contradictions relevées dans la littérature sur la participation relative de l'intestin et du foie à la sécrétion interne du cholestérol sont plus la conséquence de différences dans les conditions expérimentales et nutritionnelles (âge et sexe des animaux, composition du régime, moment du nycthémère, ...) que de la nature du précurseur utilisé (^3H_2O, ^{14}C acétate, ...). En effet, une étude comparative de l'incorporation d'eau tritiée et d'acétate ^{14}C dans les stérols des entérocytes a montré un gradient décroissant radioactif crypte-villosité, identique pour les 2 précurseurs, quel que soit le site de l'intestin étudié (duodénum, jéjunum ou iléum) validant ainsi l'emploi de l'acétate ^{14}C. D'autres expériences, cependant, ont prouvé l'existence de différences tissulaires dans la dilution cytosolique de l'acétyl CoA ^{14}C par l'acétyl CoA endogène chez des rats nourris d'un aliment témoin enrichi ou non en cholestyramine ou encore en nutrition parentérale. Après infusion intraveineuse d'acétaté 1-2-^{13}C, l'enrichissement isotopique ^{13}C du cholestérol nouvellement synthétisé est moindre dans le foie que dans l'intestin. La dilution de l'acétyl CoA endogène utilisé pour la synthèse du cholestérol est environ 2 fois plus forte dans l'hépatocyte que dans l'entérocyte. Cette observation est corroborée par les résultats obtenus à partir de nombreux groupes expérimentaux portant sur une large gamme de valeurs de sécrétion interne et de radioactivité des stérols dans le foie et l'intestin après administration d'acétate ^{14}C. Pour ceux-ci, il a été possible de calculer que pour une même radioactivité dans les stérols de l'intestin ou du foie correspond une sécrétion interne de cholestérol 1,9 fois plus forte dans l'intestin que dans le foie. De nombreuses données présentées dans ce travail sont en faveur du fait qu'une bonne estimation de la cholestérogénèse locale peut être obtenue chez le rat après administration* in vivo *d'acétate ^{14}C. Cependant, à cause des différences de radioactivité spécifique de l'acétyl CoA impliqué dans la synthèse du cholestérol du foie ou de l'intestin, il est important de confirmer par l'emploi d'acétate ^{13}C par exemple, que ces différences de dilution restent du même ordre de grandeur, pour une nouvelle condition expérimentale.*

^3H_2O / méthode d'équilibre isotopique / sécrétion interne / ^{13}C-acétate / enrichissement isotopique ^{13}C / dilution cytosolique / acétyl CoA

INTRODUCTION

Several *in vivo* methods have been used to estimate the rate of cholesterol synthesis in the body such as balance methods (Grundy and Ahrens, 1969) and isotope equilibrium methods (Chevallier and Lutton, 1966; Lutton and Chevallier, 1972a) or to compare the relative activity of cholesterol synthesis in the organs (intravenous or sub-cutaneous injection of tritiated water (Andersen and Dietschy, 1979; Jeske and Dietschy, 1980; Stange and Dietschy,

1983; Perrodin and Lutton, 1985) [^{14}C]acetate (Andersen and Dietschy, 1979; Perrodin and Lutton, 1985), [^{14}C]mevalonate (Liu *et al,* 1975) HMG CoA reductase activity measurement (Dugan *et al,* 1972; Shapiro and Rodwell, 1972) and desmosterol inhibition studies (Gibbons and Pullinger, 1977; 1979). However, the most widespread easiest to use method remains the measurement of *in vivo* incorporation of [^{14}C] acetate into organ sterols. This method has been subject to various criticisms (Andersen and Dietschy, 1979; Jeske and Dietschy, 1980; Férézou *et al,* 1986) since possible local differences in the labeling of the cholesterol precursor (acetyl CoA) prevent the accurate assessment of absolute cholesterol synthesis rates. Consequently, a number of authors now prefer to use ^3H_2O (Edwards *et al,* 1972; Andersen and Dietschy, 1979; Turley *et al,* 1981; Stange and Dietschy, 1983) because the isotope equilibrium of water is reached rapidly in the whole body and its specific radioactivity can be determined easily. This last method, however, has the serious disadvantage of using very large quantities of radioactivity per animal (Férézou *et al,* 1986) and therefore requires extremely strict experimental procedures. A critical analysis of the use of [^{14}C]acetate is presented here, taking into account over 100 experiments carried out in our laboratory and some other studies concerning *in vivo* cholesterol synthesis.

MATERIALS AND METHODS

Animals

Rats of the Wistar strain were maintained in an animal house with controlled light cycle (8–20 h), temperature (24 ± 1 °C) and hygrometry. After weaning, the rats were fed *ad libitum* a semi-purified diet (Chevallier *et al,* 1975) which had the following percent composition: sucrose 53, casein 23, salts 5, yeast 2.3, milk 4, agar–agar powder with added vitamins 2.5, nut oil 0.8, lard 9.2, cystine 0.2, cholesterol 0.015. When they were 3 months old, they received the experimental diet for 2 ([^{14}C]acetate experiments) or 4 months (isotope equilibrium experiments).

Isotope experiments

[^{14}C]Acetate

3.7 to 7.4 MBq of sodium 1-[^{14}C]acetate (1.66 – 2.22 GBq/mmol) dissolved in a 0.2 ml normal saline solution were injected subcutaneously and the rats were killed by intra-aortic puncture 70 min later under pentobarbital anesthesia. Blood, liver and small intestine were collected.

^3H_2O

4.85 GBq of tritiated water (400 MBq/ml) was given by intravenous injection into the penile vein and the rats were killed 70 min, 8 or 24 h later. The liver, stomach, small intestine, caecum and colon were collected after exsanguination and after washing the cardiovascular system several times with saline. The small intestine was divided into 4 equal quarters. They were initially washed with a first solution (NaCl 154 mM, dithiothreitol 1 mM), and then with a second solution (NaCl 96 mM, KCl 1.5 mM, sodium citrate 27 mM, KH_2PO_4 8 mM, Na_2HPO_4 5.6 mM, pH 7.3) for 15 min in a water bath at 37 °C according to Weiser (1973). Sequential fractions of isolated enterocytes were obtained by a series of incubations and repeated washings of the gut loops with a third solution (NaCl 137 mM, KCl 2.69 mM, Na_2HPO_4 9.10 mM, KH_2PO_4 1.47 mM, EDTA 1.5 mM, dithiothreitol 0.5 mM, pH 7.4) at 37 °C. These fractions correspond to the cells from the top (fractions 1–3) or the middle (fractions 4–6) of the villus and to the crypt cells (fractions 7–9).

Isotope equilibrium method

The theory and application of this method has been published (Chevallier and Lutton, 1966;

Lutton and Chevallier, 1972a). Briefly, 2 isotope equilibria were achieved by daily subcutaneous injections (1,2-[^{3}H]cholesterol, 0.2 mg, 37 kBq/day) and feeding (4-[^{14}C]cholesterol, 9 kBq/100 g diet). Isotope administration continued for 2 months. Urine and feces were collected during the last 4 weeks of the experiment. At the end of the 8th week, the animals were anesthetized with pentobarbital and blood was collected by aortic puncture. The liver was removed after washing the cardiovascular system with isotonic saline. Heparinized blood samples were centrifuged (2 200 *g*, 20 min, 4 °C) and plasma and red cells were recovered. The radioactivities of neutral fecal sterols and the specific radioactivities of dietary and exchangeable cholesterol (plasma, red blood cells, liver) were used to calculate the rates of absorption, internal and fecal external secretions and fecal and urinary excretions (Chevallier and Lutton, 1966; Lutton and Chevallier, 1972a). Internal secretion is ensured by entry into the plasma of cholesterol synthesized within the organs (Lutton, 1976). External secretion represents elimination in the feces of cholesterol synthesized in the intestine (Lutton, 1976).

[^{13}C]Acetate intravenous infusion

The animals received an isotonic solution of sodium 1,2[^{13}C]acetate through a polyethylene catheter (0.025 in ID) previously inserted under light anesthesia into the superior vena cava *via* the jugular vein, one day before. The intravenous infusion lasted 4 h, at a constant rate of 1.2 ml/h (Braun Secura perfusor). Animals were killed as above and blood, liver and small intestine were collected.

Chemical and isotopic techniques

Sodium 1–[^{14}C] and 1,2–[^{13}C]acetate, $^{3}H_2O$, 4–[^{14}C] and 1,2–[^{3}H] cholesterol were purchased from the CEA (France). Lipids of the intestinal cells, intestinal walls, red blood cells, plasma and liver were saponified in boiling alcoholic 2 N KOH for 2 h. The sterols were extracted with petroleum ether and precipitated with digitonin by the Sperry and Webb method (Sperry and Webb, 1950). After washing, the digitonide complexes were weighed, then broken down with pyridine. Cholesterol was purified from its possible labeled sterol precursors by thin-layer chromatography on alumina gel, impregnated with $AgNO_3$ with a chloroform/petroleum ether/acetone mixture (60/30/2, v/v/v) as migrating solvent or by HPLC on a Lichrosorb Si 60–7 column (Chrompack) eluted with a mixture of hexane/isopropanol containing 0.7% water (95:5, v/v) (Sulpice and Férézou, 1984). Cholesterol was assayed by the Lieberman-Burchard reaction. Radioactivity was measured in a PPO/dimethyl–POPOP/toluene solution with a liquid scintillation spectrometer (MR 300, Kontron) in glass flasks with a low background (Mathé *et al*, 1977).

The ^{13}C labeling pattern of free cholesterol samples was analyzed by gas chromatography–mass spectrometry (GC–MS) with selected ion monitoring. The quadruple mass-spectrometer (Ribermag 10–10) coupled to a PDP8 computer was connected to a gas chromatograph (Intersmat IGC 120) equipped with a glass capillary column as previously described (Férézou *et al*, 1986). Ion selection was monitored on M(*m/z* 386) and the additional molecular ions. The relative amounts of molecules weighing M + 1, M + 2, ... were calculated after correction for the natural abundance of these molecules, measured by the same method on standard cholesterol. The percent distribution of ^{13}C atoms according to the molecular weight of the newly synthesized labeled cholesterol (M + 1 to M + 11) was calculated by a basic computer program.

RESULTS

Results obtained by 2 methods: isotope equilibrium and ^{14}C acetate incorporation

The results of 22 isotope equilibria and 23 [^{14}C]acetate experiments (4–8 rats per group) are shown in table I. The 2 methods were simultaneously applied under 13 experimental conditions. When internal cholesterol secretion was low (cholesterol (CH) or orotic acid (O) in the diet, semi-purified diet (C)), the sterol radioactivity in

the liver and intestine was also low; under conditions which strongly stimulate internal secretion (tristearin (ST) or tripalmitin (PA) diet, cholestyramine treatment (CL), common bile duct ligation (L), commercial diet (GP)), the sterol radioactivity in these 2 organs increased markedly. A significant positive correlation was found between the rates of internal secretion and the sum of the radioactivities of the hepatic and intestinal sterols, measured 70 min after [^{14}C]acetate administration, in all of the experimental groups (N = 13, r = 0,80, P < 0.001, y = 0.95 + 0.85.10^{-4}x; (fig 1). In fact, a better correlation was observed between the rates of internal secretion and the radioactivities of liver sterols (for N = 13, r = 0.89, P < 0.001, y = 9.4 + 1.45.10^{-4} x) with a minimal value of the cholesterol internal secretion (ordinate at the origin) which reached 9.4 mg/day (fig 2). Under the same conditions, internal secretion was not correlated with the radioactivity of intestinal sterols (fig 2).

Results obtained with two cholesterol precursors: tritiated water and ^{14}C acetate

A comparative study of tritiated water and [^{14}C]acetate incorporation into the liver and intestinal sterols was carried out in rats fed a semi-purified diet containing 0.5% cholesterol. Only the results concerning the topological study of intestinal cholesterol synthesis have been previously analyzed (Perrodin and Lutton, 1985).

The sterol radioactivity was measured in the liver, small intestine and plasma of rats killed 70 min (N = 2), 8 h (N = 1) or 24 h (N = 1) after intravenous injection of tritiated water (4.85 GBq/rat) and of animals killed 70 min (N = 4), 8 h (N = 6) or 24 h (N = 3) after subcutaneous injection of [^{14}C] acetate (3.7 MBq) (fig 3). Whatever the precursor or time considered, the radioactivity of intestinal sterols was always much higher than that of liver sterols,

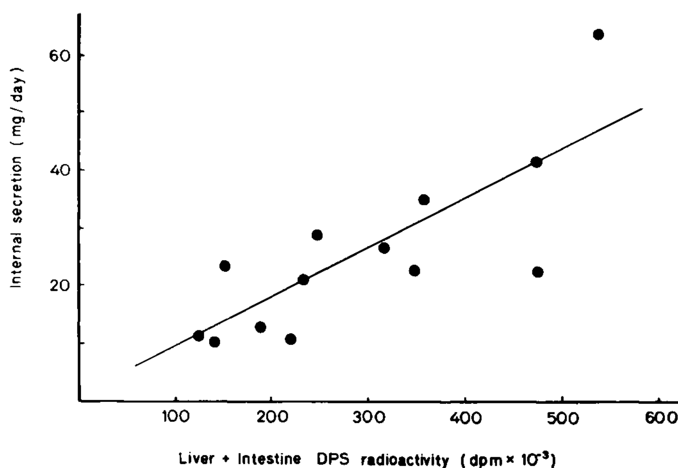

Fig 1. Internal secretion (mg/day) *versus* radioactivity of digitonin-precipitable sterols in the liver plus intestine (dpm per 100 µCi or 3.7 MBq [^{14}C]acetate). The experiment abbreviations are explained in the Table I footnote; C, CYS; CYS-T, PA, MYR, L, CL, SQ2, CH, LYS, GP, O, ST; r = 0.80, P < 0.001, y = 0.95 + 0.85.10^{-4} x.

Table I. Critical analyses.

Experiment	Internal secretion (mg/day)	Sterol radioactivity dpm per 100 μCi [¹⁴C]acetate Liver	Intestine	Ref
C	13.0 ± 0.5	32 272 ± 6557	156 000 ± 13 825	Rukaj and Sérougne, 1983
CYS	23.7 ± 3.9	53 606 ± 13 382	97 356 ± 12 046	Rukaj and Sérougne, 1983
CYS-CH	11.8 ± 1.3			Rujak and Sérougne, 1983
CYS-T	35.0 ± 2.8	210 901 ± 52 422	146 182 ± 5797	Rujak and Sérougne, 1983
U	12.5 ± 0.6			Lutton *et al*, 1980
OL	14.4 ± 1.2			Lutton *et al*, 1980
PA	28.4 ± 4.6	56 968 ± 16 069	186 883 ± 8607	Lutton *et al*, 1980
ST	22.3 ± 0.7	140 858 ± 27 892	332 892 ± 26 890	Lutton *et al*, 1980
ER	33.2 ± 2.8			Lutton *et al*, 1980
CAP	11.7 ± 0.4			Lutton *et al*, 1983
MCT	13.8 ± 0.3			Lutton *et al*, 1983
LAU	15.4 ± 1.1			Lutton *et al*, 1983
MYR	21.1 ± 1.4	64 193 ± 28 419	166 747 ± 21 386	Lutton *et al*, 1983
L	64.2 ± 2.7	282 340 ± 16 014	252 377 ± 39 719	Lutton *et al*, 1973
CL	41.7 ± 1.8	253 577 ± 48 477	218 147 ± 39 870	Lutton *et al*, 1973
SQ		51 012 ± 23 648	153 457 ± 35 933	
SQ2	11.3 ± 0.8	4815 ± 754	119 904 ± 8623	
CH	10.6 ± 1.2	5000 ± 1000	136 304 ± 10 000	
LYS	22.7 ± 2.9	115 063 ± 57 068	232 370 ± 29 716	
GP	26.6 ± 1.9	148 405 ± 23 902	165 760 ± 9634	Mathé *et al*, 1977
O	10.8 ± 0.5	66 626 ± 4075	152 882 ± 14 908	Lutton *et al*, 1986
APP			113 629 ± 18 950	Lutton *et al*, 1986
2-4-D		28 020 ± 11 070	163 849 ± 27 230	Ohta *et al*, 1987
PCA	11.2 ± 0.7			Magot *et al*, 1983
MCA	14.2 ± 0.8			Magot *et al*, 1983
F		53 558 ± 15 101	185 173 ± 14 401	
C-O		61 213 ± 10 266	222 492 ± 26 784	
C-12		22 338 ± 3746	232 727 ± 14 219	
MGP-0		240 200 ± 54 490	131 682 ± 16 635	
MC-12		32 292 ± 2052	194 746 ± 18 914	
FGP-0		162 494 ± 20 817	97 980 ± 8892	
FC-12		39 570 ± 7381	209 315 ± 18 134	

C: semi-purified control diet; CYS: control diet with 5% cystine at the expense of sucrose; CYS-CH: control diet with both 5% cystine and 1% cholesterol; CYS-T: control diet with 5% cystine in which lard was replaced by tristearin; semi-purified diets containing 20% lard (U), triolein (OL), tripalmitin (PA), tristearin (ST), or trierucin (ER); semi-purified diets containing 10% tricaproin (CAP), medium-chain triglycerides (MCT), trilaurin (LAU) or trimyristin (MYR); L: rats with experimental ligation of bile duct; CL: control diet with 2% cholestyramine; SQ: control diet with 0.02% squalene; SQ2: control diet with 0.2% of squalene; CH: control diet with 0.5% cholesterol; LYS: control diet with 10% lysine at the expense of sucrose; GP: commercial stock diet (Extralabo, Pietrement, Sainte-Colombe, 77160 Provins, France); O: control diet with 2% orotic acid; APP: 48 h APP treatment in fasting rats; 2-4-D: control diet with 0.04 nmol/kg of dichlorophenoxyproprionic acid; PCA: rats with portocaval anastomosis; MCA: rats with mesentericocaval anastomosis. (C to MCA: adult male rats killed at 10 am). F: adult female rats fed the control diet and killed at 10 am; C–0: adult male rats fed the control diet and killed at midnight; C-12: adult male rats fed the control diet and killed at noon; MGP-0: young male rats fed with a commercial diet and killed at midnight; MC-12: young male rats fed with the control diet and killed at noon; FGP-0: young female rats fed a commercial diet and killed at midnight; FC-12: young female rats fed the control diet and killed at noon. Results are expressed as mean ± SEM.

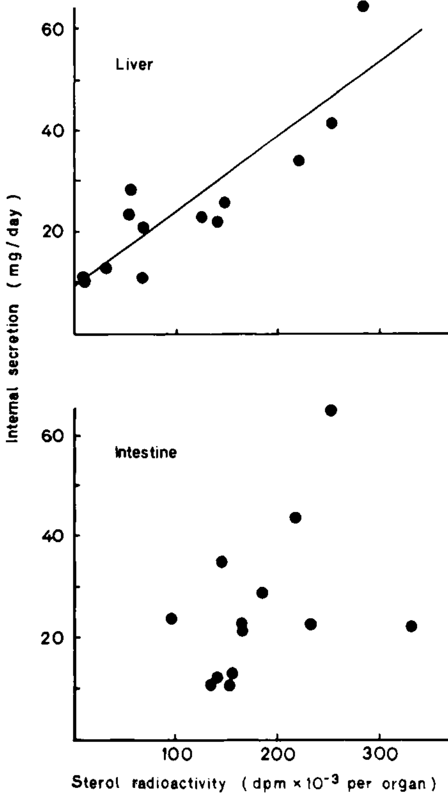

Fig 2. Internal secretion (mg/day) *versus* radioactivity of digitonin-precipitable sterols in the liver or intestine (dmp per 100 μCi or 3.7 MBq [^{14}C]acetate). C, CYS, CYS-T, PA, MYR, L, CL, SQ2, CH, LYS, GP, O, ST; $r = 0.89$, $P < 0.001$, $y = 9.4 + 1.45.10^{-4} x$.

which, in turn, was higher than that of plasma cholesterol. After the [^{14}C]acetate pulse, radioactivity of the intestinal sterols decreased monoexponentially over a period of time, demonstrating the renewal of newly synthesized cholesterol, whereas that of the plasma and liver sterols increased over a period of 24 h (Perrodin and Lutton, 1985). Following an intravenous injection of tritiated water, an isotope equilibrium of the body water was obtained after 10 min (Jeske and Dietschy, 1980) and was maintained for 24 h (author's unpublished observations). Consequently, the sterol radioactivity increased during this period in the intestine, liver and plasma. When the specific radioactivities of cholesterol in the isolated enterocytes were compared according to their location on the villus (crypt-top) and their level in the intestine (duodenum, jejunum, proximal and distal ileum), taking as reference that of the duodenal crypt enterocytes, similar results were obtained with the 2 precursors 70 min after [^{14}C]acetate pulse or within the 24-h period following ^3H_2O injection (fig 4). The cholesterol labeling decreased from the duodenum to the proximal ileum and increased in the distal

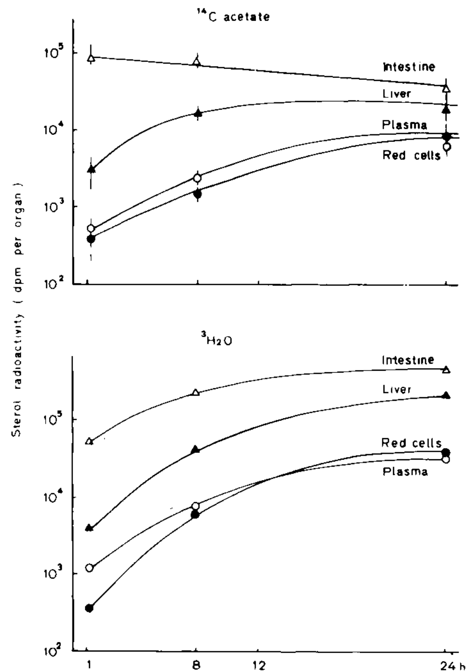

Fig 3. Variation in radioactivity of digitonin-precipitable sterols in the intestine, liver, plasma and red blood cells during 24 h after [^{14}C]acetate or ^3H_2O administration in rats fed a cholesterol rich semi-purified diet.

ileum. Furthermore, whatever the intestinal segment considered and the precursor injected, the specific radioactivity of cholesterol decreased from the villus crypt cells towards the top cells.

Isotope enrichment of acetyl CoA, precursor of cholesterol in the liver and intestine

Taking into account the criticisms concerning the use of [^{14}C]acetate, we thought it was necessary to investigate possible differences in the isotope labeling of the acetyl CoA used for cholesterol synthesis, according to the organs, which could account for some of the differences between the results obtained with [^{14}C]acetate and tritiated water.

Male rats fed the control semi-purified diet (fig 5a), or this diet with 2% cholestyramine (fig 5b) and male rats under total parenteral nutrition (fig 5c) received an intravenous infusion of 1,2[^{13}C]acetate. On the basis of the previous results, it can be assumed that the dose of [^{13}C]acetate infused does not have a mass effect on the rate of incorporation of exogenous acetate into cholesterol (Férézou *et al,* 1986). The use of the stable isotope has the advantage of enabling the proportion of newly synthesized labeled molecules to be calculated according to their molecular weight (M + 1 to M + 11). This analysis directly reflects the ^{13}C-enrichment of acetyl CoA used for cholesterol synthesis. The distribution of ^{13}C atoms (% ^{13}C) in the newly synthesized cholesterol isolated from the intestinal mucosa, liver and plasma is illustrated in figure 5. Under the 3 conditions tested, about 3/4 of the ^{13}C atoms incorporated into cholesterol were found in molecules of weight ≥ M + 4 in the intes-

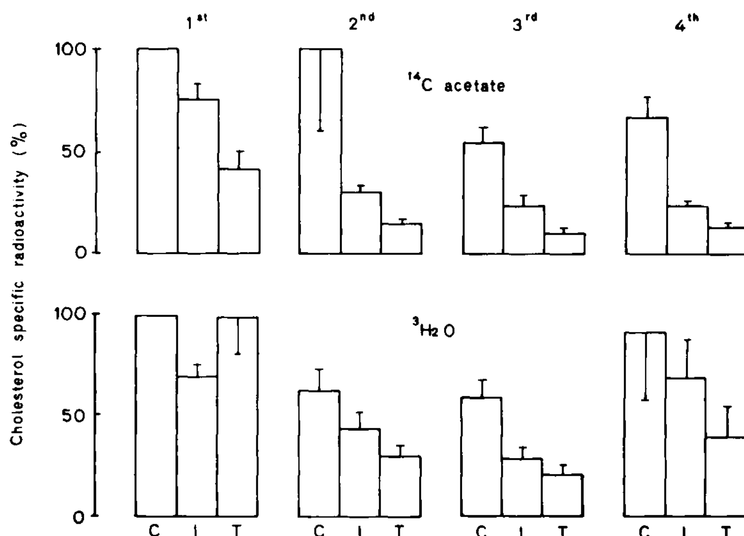

Fig 4. Cholesterol specific radioactivity in the enterocytes of rats fed a cholesterol-rich semi-purified diet and killed 70 min after a subcutaneous injection of [^{14}C]acetate (*N* = 4) or killed 70 min, 8 or 24 h after an intravenous injection of $^{3}H_2O$ (*N* = 4). The cells were collected after dividing the small intestine into 4 equal quarters (1st, 2nd, 3rd, 4th). C : crypt cells; I : intermediate cells; T: top cells. The values (mean ± SEM) are the percentage of cholesterol specific radioactivity in the duodenal crypt cells.

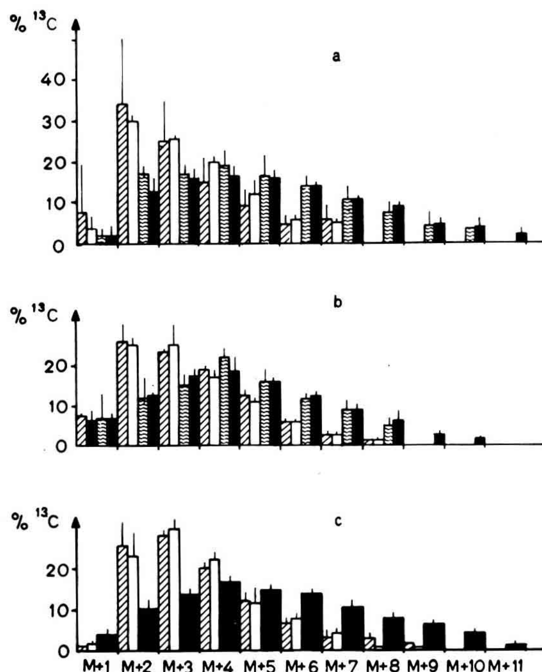

Fig 5. Distribution of ^{13}C atoms (% ^{13}C) according to the molecular weight of biosynthetized cholesterol in plasma ▨, liver ▭, intestinal wall ▤ and mucosa ■ or intestinal wall + mucosa ▩ in male rats (a), cholestyramine-fed rats (b) and parenterally fed rats (c) after infusion of [^{13}C]acetate during 4 h (N = 4). Results are expressed as mean values.

tine and only 1/3 in the liver and plasma. Cholesterol was therefore synthesized from acetyl CoA, which was significantly more ^{13}C-enriched in the intestine than in the liver.

DISCUSSION

The most common method of estimating tissue cholesterol synthesis involves measuring the *in vitro* or *in vivo* incorporation of radioactive acetate into the organ sterols. This method, however, can be criticized, especially in *in vivo* experiments (Andersen and Dietschy, 1979; Jeske and Dietschy, 1980; Férézou *et al*, 1986). Nevertheless, it has the advantage over tritiated water of being simple and requiring less radioactivity. However, regardless of the precursor used, the radioactivity incorporated into the tissue sterols after a relatively short time (usually 1 h) is an index of the level of cholesterol synthesis at a given time of the circadian cycle. In contrast, the sterol balance as well as the isotope equilibrium methods (Chevallier and Lutton, 1966; Lutton and Chevallier, 1972a), which have been used under extremely varied nutritional and experimental conditions (Lutton and Chevallier, 1972b, 1972c; Lutton *et al*, 1973; Mathé *et al*, 1977; Lutton *et al*, 1980; Lutton *et al*, 1983; Magot *et al*,

1983; Rukaj and Sérougne, 1983; Lutton *et al,* 1986; Ohta *et al,* 1987), allow cholesterol internal secretion to be measured over a period of several days so that the mean rate of cholesterol synthesis over 24 h can be assessed. We first compared the results obtained with these 2 techniques under various physiological conditions and then compared them to published data.

Daily cholesterol internal secretion in an adult male rat, fed a semi-purified diet based on 53% sucrose and 9% lard (Chevallier and Lutton, 1966) reached an average of 13 mg. It increased from 25 to 100% when lard was replaced by trimyristin (Lutton *et al,* 1983), tripalmitin, tirstearin or trierucin (Lutton *et al,* 1980). It doubled or even tripled when cholestyramine was added, after common bile duct ligation (Lutton *et al,* 1973) or even after replacing the semi-purified diet by a fiber-rich commercial diet (GP) (Mathé *et al,* 1977). It decreased to 8–11 mg/day after adding to the diet cholesterol (Mathé and Chevallier, 1979), orotic acid or squalene (table I), sodium taurocholate (Lutton *et al,* 1973) or after porta-caval anastomosis (Magot *et al,* 1983; Bendjellit *et al,* 1985) and in the aged male rat (Lutton and Chevallier, 1972b). In the adult female rat, internal secretion was less than 9 mg/day, but multiplied by a factor of 2 or 3 during gestation and lactation (Lutton and Chevallier, 1972b).

This study shows that a strong linear correlation exists between the internal secretion of cholesterol and the sterol radioactivity measured in the liver 70 min after [^{14}C]acetate injection. The ordinate at the origin corresponds to an internal cholesterol secretion of ≈ 10 mg/day. This basal internal secretion can be attributed, in a first approximation, to the intestinal participation in the total internal secretion. A similar value of about 10 mg/day was also calculated from experiments using the isotope equilibrium method (Chevallier and Lutton, 1973; Lutton and Chevallier, 1973), intravenous infusion of [^{14}C]acetate (Chevallier and Magot, 1975; Magot and Chevallier, 1979) or by kinetic studies analyzed by modelling of the rat cholesterol system (Magot, 1989). The fact that a similar value of 10 mg/day for the intestinal participation in the internal secretion of cholesterol was found by different methods indicates that the results obtained 70 min after [^{14}C]acetate administration at 10 am gave a good index of cholesterol synthesis.

After adding 0.5% cholesterol to the diet of an adult male rat, internal secretion, measured by the isotope equilibrium method reached 9 to 10 mg/day (Mathé and Chevallier, 1979); 70 min after [^{14}C]acetate injection, the radioactivity of intestinal sterols decreased by 30–40% when compared to that observed in control rats whereas that of hepatic sterols was virtually nil. A very similar situation was observed after tritiated water administration under the same dietary conditions. The intestine therefore appeared to be the major site of internal secretion. ^{3}H or ^{14}C data indicate the same difference in the relative activity of enterocytes to produce cholesterol (fig 4). These results agree with the HMGCoA reductase activity measured in the same organs (Sugano *et al,* 1982). According to the location of the enterocytes on the villus and the segment (proximal or distal) of the intestine considered, tritiated water incorporation by the sterols was between 2–10 μmol/mg of protein/h as in Stange and Dietschy's *in vivo* data (Stange and Dietschy, 1983). In the whole intestine of adult male rats, the hourly incorporation of water into the digitonin-precipitable sterols would be between 2–3 μmol; these values are close to those found by Turley *et al* (1981) or Feingold *et al* (1983) in young female rats. The values of water incorporation into the liver sterols were significantly

lower in adult rats fed 0.5% exogenous cholesterol (0.2–0.5 μmol) than in young rats fed a commercial diet with 2% cholesterol for 4–8 days (Turley *et al*: 0.7 ± 0.2; Feingold *et al*: 1.3 ± 0.1 μmol). If we estimate, according to Jeske and Dietschy (1980) that an average of 12.4 mol of water are incorporated per mol of cholesterol synthesized, the total hepatic and intestinal cholesterol synthesis calculated with tritiated water (about 0.3 $\mu mol \cdot h^{-1}$) was 3 times lower than that of internal secretion measured by the isotope equilibrium method. Nevertheless, the radioactivity ratio (intestine/liver) under these conditions was between 6 to 10, 1 or 8 h after tritiated water administration as described by these authors (Turley *et al,* 1981; Feingold *et al,* 1983). Under the same conditions, but after acetate administration, we observed a ratio of 6 to 20. These observations underline the fact that the results obtained with the 2 isotopes are far from being contradictory. In fact, the differences between Dietschy's conclusions and our own concerning the relative part played by the intestine and liver in cholesterol synthesis are due to differences in physiological and dietary conditions rather than to the different techniques used. For Turley *et al* (1981) and many other workers, the "control" animal was a young female rat (from 150–200 *g*), fed a commercial diet, while ours was an adult male rat (400 g) fed a semi-purified diet with 9% lard and 53% sucrose. After injecting [^{14}C]acetate into this animal, the ratio of the radio-activity of intestinal sterol to that of liver sterols ranged from 10 (during the day), to 3.6 during the nocturnal peak of cholesterol synthesis (table I). When we used young female rats weighing 150–200 g, the intestine then incorporated 5–6 times more [^{14}C]acetate in their sterols than the liver, during the light phase in rats fed a semi-purified diet, and only half as much during the nocturnal peak in rats fed a commercial diet. Thus the radioactivity ratio (intestine/liver) was higher in a male adult animal during the entire circadian cycle than in a young animal and higher in an animal fed a semi-purified diet than in one fed a commercial diet.

It is noteworthy that the intestine/liver ratios of sterol radioactivity varied generally in parallel but they were significantly higher after [^{14}C]acetate than after $^{3}H_2O$ administration. Such a difference might be interpreted as resulting from a different isotopic enrichment of the acetyl CoA in the liver and intestine. We therefore investigated this possibility by analyzing the isotope labeling pattern of newly synthesized cholesterol in these 2 tissues after a [^{13}C] acetate infusion. Our results clearly show that the dilution of the labeled acetyl CoA precursor of cholesterol by the endogenous substrate was significantly higher (about double) in the hepatocytes than in the enterocytes. A corrective factor must be introduced in order to compare hepatic and intestinal cholesterol synthesis on the basis of local [^{14}C]acetate incorporation into sterols. Nevertheless, under 3 experimental conditions susceptible to induce some modifications of the pool and/or the production rate of acetyl CoA in these 2 organs of the adult male rat, no important change in the relative distribution of the ^{13}C atoms in synthesized cholesterol was observed (fig 5). Moreover, results obtained with several experimental groups (CH, C, LYS, CYS, GP, CYS-T, CL and L) exhibiting a large range of internal secretion values (from 10.6 for the CH group to 64.2 mg/day for the L group) (isotope equilibrium method) and sterol radioactivities for liver and intestine (100 μCi [^{14}C]acetate injection), allowed us to calculate that a sterol radioactivity of 100 000 dpm corresponded to a cholesterol internal secretion of 11.5 ± 1.0 mg/day for the liver and 5.9 mg/day for the intestine. This shows that the liver/intestine [^{14}C]acetate dilution ratio reached 1.9, as shown by the [^{13}C]acetate

experiments. Furthermore, since the ^{14}C-radioactivity of intestinal sterols remained between 150 000–200 000 under most of the experimental conditions, the "basal" internal secretion of the intestine was always therefore between 8.8 and 11.8 mg/day, as in the previous deductions (fig 2). Several cases, however, did not fit into the framework of this analysis: it is interesting to note that they concern rats fed homogeneous long-chain fatty acid triglycerides, trimyristin (MYR), tristearin (ST) and tripalmitin (PA). Feeding a large quantity of long-chain fatty acids probably increases the amount of acetyl CoA available in the liver and intestine for cholesterol synthesis. These examples underline the limitations of the use of [^{14}C]acetate in estimating tissue cholesterol synthesis.

This study has enabled us to define the conditions under which the ^{14}C-acetate incorporation into sterols can be used to validly estimate *in vivo* rat cholesterol synthesis. In many physiological situations, the results obtained with this method are similar to those obtained with tritiated water and are well correlated with results from the isotope equilibrium method. Nevertheless, certain situations which disturb the acety-CoA pool and metabolism, particularly adding large quantities of long-chain fatty acids to the diet, can make the results uncertain. For this reason, other methods ([^{13}C]acetate for example) must be used to make sure that the dilution by the endogenous substrate of the labeled acetyl-CoA used for cholesterol synthesis in the liver and intestine is not modified markedly.

REFERENCES

Andersen JM, Dietschy JM (1979) Absolute rates of cholesterol synthesis in extra hepatic tissues measured with ^{3}H labeled water and ^{14}C labeled substrates. *J Lipid Res* 20, 740-752

Bendjellit S, Mathé D, Castaing D (1985) Cholesterol metabolism in lean and obese Zucker rats: effects of portocaval anastomosis. *Int J Obesity* 9, 1-9

Chevallier F, Lutton C (1966) Vitesses des processus de renouvellement du cholestérol contenu dans son espace de transfert chez le rat. I. Méthodes et résultats. *Bull Soc Chim Biol* 48, 507-524

Chevallier F, Lutton C (1973) The intestine is the major site of cholesterol synthesis in the rat. *Nature New Biol* 242, 61-62

Chevallier F, Magot T (1975) Evidence of major role of the intestine in cholesterol synthesis in the adult male rat. *Experientia* 31, 627-629

Chevallier F, Serougne C, Champarnaud G (1975) Effect upon brain weight and cholesterol content of maintaining rats of various ages at constant weight. *J Nutr* 105, 1 003-1 011

Dugan RE, Slakey LL, Briedis AV, Porter JW (1972) Factors affecting the diurnal variation in the level of β-hydroxy-β-methylglutaryl CoA reductase and cholesterol synthesizing activity in rat liver. *Arch Biochem Biophys* 152, 21-27

Edwards PA, Muroya H, Gould RG (1972) *In vivo* demonstration of the circadian rhythm of cholesterol biosynthesis in the liver and intestine of the rat. *J Lipid Res* 13, 396-401

Feingold KR, Wiley MH, Macrae G, Lear S, Moser AH, Zsimond G, Siperstein MD (1983) *De novo* sterologenesis in the intact rat. *Metabolism* 32, 75-81

Férézou J, Sulpice JC, Lutton C (1986) Evidence for different isotopic enrichments of acetyl CoA used for cholesterol synthesis in the liver and intestine: a study in the rat by mass fragmentography after intravenous infusion of ^{13}C acetate. *Biochim Biophys Acta* 875, 227-235

Gibbons GF, Pullinger CR (1977) The use of cholesterol oxidase in an improved method for measurement of the true rate of cholesterol synthesis. *Biochem Soc Trans* 5, 1 003-1 004

Gibbons GF, Pullinger CR (1979) Utilisation of endogenous and exogenous source of sub-

strate for cholesterol biosynthesis by isolated hepatocytes. *Biochem J* 177, 255-263

Grundy SM, Ahrens EH (1969) Measurements of cholesterol turnover synthesis and absorption in man carried out by isotope kinetic and sterol balance methods. *J Lipid Res* 10, 91-107

Jeske DJ, Dietschy JM (1980) Regulation of rates of cholesterol synthesis *in vivo* in the liver and carcass of the rat measured using (^3H) water. *J Lipid Res* 21, 364-376

Liu GCK, Ahrens EH, Schreibman PH, Samuel P, Mcnamara DJ, Crouse JR (1975) Measurement of cholesterol synthesis in man by isotope kinetics of squalene. *Proc Natl Acad Sci USA* 72, 4612-4616

Lutton C (1976) The role of the digestive tract in cholesterol metabolism. *Digestion* 14, 342-356

Lutton C, Chevallier F (1972a) Vitesses des processus de renouvellement du cholestérol contenu dans son espace de transfert chez le rat. III. Modification et étude critique de la méthode d'équilibre isotopique. *Biochim Biophys Acta* 255, 762-779

Lutton C, Chevallier F (1972b) Vitesses des processus de renouvellement du cholestérol contenu dans son espace de transfert chez le rat. IV. Influence de l'âge, du poids, du sexe, de la gestation et de la lactation. *Biochim Biophys Acta* 280, 116-130

Lutton C, Chevallier F (1972c) Vitesses des processus de renouvellement du cholestérol contenu dans son espace de transfert chez le rat. V. Influence des proportions de glucides, lipides et protides dans l'alimentation. *Biochim Biophys Acta* 280, 131-144

Lutton C, Chevallier F (1973) Coefficient d'absorption du cholestérol alimentaire, paramètre fondamental de l'équilibre dynamique du cholestérol chez le rat. *Biomedicine* 24, 205-208

Lutton C, Magot T, Chevallier F (1980) Effect of dietary long chain fatty acids on the rates of cholesterol turnover processes, cholesterol origin and distribution in the rat intestinal lumen. *Reprod Nutr Dev* 20, 1 467-1 479

Lutton C, Magot T, Mathé D, Sulpice JC, Chevallier F (1983) Influence des acides gras à chaîne courte ou moyenne sur la dynamique du cholestérol chez le rat. *Ann Nutr Metab* 27, 162-171

Lutton C, Mathé D, Chevallier F (1973) Vitesses des processus de renouvellement du cholestérol contenu dans son espace de transfert chez le rat. VI. Influence de la ligature du cholédoque et de l'ingestion d'acides biliaires ou de cholestyramine. *Biochim Biophys Acta* 306, 483-496

Lutton C, Perrodin M, Cardona-Sanclemente LE, Serougne C (1986) *In vivo* cholesterol synthesis by the rat digestive tract. III. Evaluation of modulating factors. *Reprod Nutr Dev* 6, 1 241-1 253

Magot T (1989) A multicompartmental model of cholesterol metabolism in rats. *J Physiol* 83, 80-94

Magot T, Castaing D, Mathé D, Bismuth H, Chevallier F (1983) Effect of portocaval or mesentericocaval anatomosis on cholesterol metabolism in rats. *Atherosclerosis* 46, 77-86

Magot T, Chevallier F (1979) Measurement of the rate of cholesterol synthesis in various organs of the rat *in vivo*. *Ann Biol Anim Biochim Biophys* 19, 1 757-1 770

Mathé D, Chevallier F (1979) Effect of level of dietary cholesterol on the dynamic equilibrium of cholesterol in rats. *J Nutr* 109, 2 076-2 084

Mathé D, Lutton C, Rautureau J, Coste T, Gouffier E, Sulpice JC, Chevallier F (1977) Effects of dietary fiber and salt mixtures on cholesterol metabolism in rats. *J Nutr* 107, 466-474

Ohta F, Gaudemer F, Lutton C (1987) Hypocholesterolemic activity of racemic dichlorophenoxy propionic acid or its enantiomers in rats. *Bull Environ Contam Toxicol* 39, 966-973

Perrodin M, Lutton C (1985) *In vivo* cholesterol synthesis by the rat digestive tract. I. A topological study. *Reprod Nutr Dev* 25, 647-657

Rukaj A, Sérougne C (1983) Effect of excess dietary cystine on the biodynamics of cholesterol in the rat. *Biochim Biophys Acta* 753, 1-5

Shapiro DJ, Rodwell VN (1972) Fine structure of the cyclic rhythm of 3-hydroxy-3-methylglutaryl CoA reductase. Differential effects of cholesterol feeding and fasting. *Biochemistry* 11, 1 042-1 045

Sperry WM, Webb M (1950) A revision of the Schoenheimer–Sperry method for cholesterol determination. *J Biol Chem* 187, 97-106

Stange EF, Dietschy JM (1983) Absolute rates of cholesterol synthesis in rat intestine *in vitro* and *in vivo*: a comparison of different substrates in slices and isolated cells. *J Lipid Res* 24, 72-82

Sugano M, Fujisaki Y, Oku H, Ide T (1982) 3-hydroxy-3-methylglutaryl CoA reductase activity in the small intestine of rats fed nonpurified and semi-purified diets. *J Nutr* 112, 51-59

Sulpice JC, Férézou J (1984) Squalene isolation by HPLC and quantitative comparison by HPLC and GLC. *Lipids* 19, 631-635

Turley SD, Andersen JM, Dietschy JM (1981) Rates of sterol synthesis and uptake in the major organs of the rat *in vivo. J Lipid Res* 22, 551-569

Weiser MM (1973) Intestinal epithelial cell surface membrane glycoprotein synthesis. An indication of cellular differentiation. *J Biol Chem* 248, 2536-2541