Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [^{3}H]water

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Abstract This study was undertaken to determine the mechanisms that regulate cholesterol synthesis in vivo and to quantitate the relative importance of the liver and extrahepatic tissues as sites for sterol synthesis. Rats were administered [^{3}H]water intravenously and killed 1 hour later. The amount of [^{3}H]water incorporated into digitonin-precipitable sterols was then measured in liver, whole blood, and the remaining tissues of the carcass. In control animals, killed at the mid-dark point of the light cycle, rates of [^{3}H]-water incorporation into sterols equaled 2290 and 103 nmol/hr per g, respectively, in the liver and carcass. Cholesterol feeding suppressed synthesis in the liver but not in the extrahepatic tissues, while fasting for 48 hr suppressed synthesis in both the liver and carcass. In fasted animals subjected to stress there was a 5-fold increase in hepatic synthesis but no change in synthesis by the extrahepatic tissues. Similarly, incorporation of [^{3}H]water into sterols by the carcass was unaffected by light cycling while the liver showed a definite diurnal rhythm. In control rats, 34.5 μmol of [^{3}H]water was incorporated into sterols by the whole animal per hour. Of this amount of sterol synthesis about 54% took place in the liver while the remaining amount occurred in the tissues of the carcass. With cholesterol feeding or fasting, or during the mid-light phase of the light cycle, synthesis in the extrahepatic tissues accounted for 69 to 90% of total body sterol synthesis.—**Jeske, D. J., and J. M. Dietschy.** Regulation of rates of cholesterol synthesis in vivo in the liver and carcass of the rat measured using [^{3}H]water. *J. Lipid Res.* 1980. **21:** 364–376.

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Since the early observations that cholesterol feeding suppresses the rate of sterol synthesis in the liver (1–3), a number of major regulatory mechanisms have been described in experimental animals and man that alter rates of cholesterol synthesis in liver, intestine, and many extrahepatic tissues (4). With few exceptions, these studies have been carried out by subjecting animals or human subjects to a variety of dietary and physiological manipulations, and then assessing changes in rates of sterol synthesis in different organs by measuring rates of incorporation of a radiolabeled precursor into cholesterol in tissue slices or biopsy specimens, or by assaying the activity of microsomal 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase). Such in vitro assays of synthetic activity are normally carried out at sufficiently high substrate concentrations that maximal synthesis rates are achieved on the assumption that, in the same tissues in vivo, it is the activity of the enzymes in the biosynthetic sequence that is rate-limiting to sterol synthesis and not the availability of acetyl CoA. This assumption, however, has never been vigorously tested. Furthermore, there are a number of major problems that are recognized as being potential sources of error in the interpretation of such in vitro assay data. Microsomal HMG CoA reductase activity, for example, may vary as much as 10-fold depending upon the conditions under which the microsomes were prepared (5–7). When tissue slices or other whole cell preparations are utilized, such factors as the degree of oxygenation, the ability of the substrate to penetrate cell membranes, and intracellular dilution of the specific activity of the acetyl CoA pool may also have profound and variable effects upon apparent rates of sterol synthesis in different tissues (8–10).

Despite these problems, however, such in vitro assay systems have provided a great deal of valuable information on both the qualitative and quantitative aspects of the regulation of cholesterol synthesis in the major organ systems of the body. For example, in species such as rat, monkey, and man, the highest rates of incorporation of [^{14}C]acetate into digitonin-precipitable sterols (DPS) per gram of tissue is usually found in liver (4, 11–13). The ileum also has relatively high

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; DPS, digitonin-precipitable sterols.

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rates of sterol synthesis, while the remaining organs of the body incorporate [^{14}C]acetate into DPS at much lower rates (11–16). As a consequence of these high apparent rates of sterol synthesis and the relatively large size of these two organs, it has been calculated that the liver and intestine account for 90 to 97% of the [^{14}C]acetate incorporated into DPS that is detectable in all of the tissues of the body under in vitro conditions (11, 12). Such findings have resulted in a major emphasis on studying the mechanisms of regulation in these two organs.

These in vitro techniques also have been used extensively to study the mechanisms of regulation of sterol synthesis in specific tissues. Cholesterol synthesis in the liver, for example, manifests a significant diurnal rhythm, is markedly enhanced by stress or by interrupting the enterohepatic circulation, and is suppressed by fasting or cholesterol feeding (4, 7, 11, 12, 15–22). In addition, hepatic sterol synthesis also is regulated by the plasma levels of major lipoproteins: in general, there is an inverse relationship between the concentration of chylomicrons and high and low density lipoproteins and the rate of sterol synthesis in the hepatocytes (23, 24). In contrast, sterol synthesis in most of the extrahepatic tissues does not manifest a diurnal rhythm, is not suppressed by circulating chylomicrons or cholesterol feeding, and is not enhanced by stress or interruption of the enterohepatic circulation (11, 12, 15, 16). Fasting does reduce synthetic activity in tissues like intestine, kidney, and spleen, and intestinal synthesis is markedly enhanced by interrupting the enterohepatic circulation (1, 14, 15). Rates of sterol synthesis in endocrine tissues such as the adrenal gland, ovary, and testis are exquisitely sensitive to circulating levels of high density lipoproteins (25, 26) while low density lipoproteins appear to be principally responsible for regulation of sterol synthesis in organs such as small intestine, colon, kidney, lung, and skin (16).

While these types of studies have led to major insights into the regulation of whole body sterol balance, two recent observations have raised questions as to the validity of some of the conclusions drawn from these investigations. First, while a diurnal rhythm in hepatic cholesterol synthesis can be readily demonstrated by in vitro assay techniques, there are conflicting reports as to whether such variations in synthetic activity occur in vivo (27–29). This discrepancy raises the possibility that changes in rates of synthesis, manifest under the conditions of in vitro assays, do not necessarily reflect changes in the rates of synthesis of sterols in any given organ under in vivo conditions. It is conceivable, for example, that substrate availability, and not the levels of the biosynthetic enzymes, is rate-limiting for sterol synthesis under certain physiological circumstances. Second, a recent detailed analysis has been made in this laboratory of the degree of underestimation of true rates of sterol synthesis in various tissues when a [^{14}C]substrate such as acetate, octanoate, or glucose is used as the radiolabeled precursor. Rates of incorporation in vitro of [^{14}C]acetate into DPS, for example, equaled approximately 50% of the true rate of synthesis in liver and intestine, but only 4% to 34% of the absolute rates found in tissues like muscle, lung, testis, ovary, adrenal gland, and skin (10). This finding raises the possibility that the quantitative importance of sterol synthesis in tissues other than liver and intestine has been grossly underestimated in all previous studies utilizing various [^{14}C]substrates as the precursor for sterol synthesis.

As a result of these recent observations, therefore, the present studies were undertaken to investigate three specific problems. First, an initial group of experiments was carried out to evaluate certain technical aspects related to the measurement of rates of sterol synthesis in vivo utilizing [^{3}H]water. Second, the effect of a variety of physiological manipulations on hepatic and carcass sterol synthesis was examined, in order to determine whether the rates of synthesis in these tissues manifested the same changes in vivo as has been shown to occur utilizing in vitro assay procedures. Third, the absolute rates of sterol synthesis in the liver and in the remaining tissues of the carcass were measured under different physiological circumstances in order to document the relative importance of sterol synthesis in extrahepatic tissues to whole animal sterol balance under in vivo conditions.

MATERIALS AND METHODS

Animal preparations

The female, Sprague-Dawley-derived rats used in these studies were purchased in the range of 150–180 g (Charles River Breeding Laboratories, Wilmington, MA) and subjected to light-cycling for approximately 2 weeks before use (15, 16, 22). During this time they were allowed free access to water and Formulab Rat Chow Diet (Ralston Purina Co., St. Louis, MO). A variety of other physiological manipulations were then utilized in order to change the rates of sterol synthesis in the liver and in the various tissues of the carcass. Groups of rats were fasted for 48 hr in gang cages or in individual restraining cages (22). This latter group has been shown in previous experiments to manifest a marked increase in hepatic cholesterol synthesis above the level seen in animals fasted in the gang cages that is presumably due to the stress associated with restraint (22). Other groups of animals were fed diets containing 2% cho-

lesterol (w/w) or 3% cholestyramine (Mead Johnson Research Center, Evansville, IN) for 4 days prior to use.

Determination of sterol synthesis rates in vivo

After a number of preliminary experiments, as described in detail in the Results section, the following standardized procedure was adopted for measuring rates of sterol synthesis in the intact rat. At the beginning of an experiment a tail vein catheter was introduced into each rat and the animal was then placed in an individual restraining cage under a fume hood. [^{3}H]-water, in amounts varying from 50 to 100 mCi contained in 0.1 to 0.2 ml of isotonic NaCl solution, was rapidly injected intravenously. The catheter was then immediately removed from the animal and the tail was taped to prevent bleeding. This whole procedure required about 10 sec and was taken as time 0 in all studies. The animal remained in the restraining cage under the hood until the experiment was terminated, usually 60 min later, and was not given any further injections of solutions nor any food or water. At the appropriate time, the animal was removed from the cage and anesthetized with ether. The abdomen was immediately opened and 5.0 ml of blood was withdrawn from the abdominal aorta into a heparinized syringe. The liver was quickly rinsed in situ, with 20 ml of cold isotonic NaCl solution through the portal vein, and removed from the body. The whole remaining carcass, including the NaCl solution used to rinse the liver, was placed in a large beaker containing alcoholic KOH and saponified on a steam bath with stirring for approximately 2–3 hr until totally dissolved. The contents of the beaker were then transferred quantitatively to a 500 ml volumetric flask and brought to volume. Aliquots of the whole blood sample (usually 1.0 ml) were transferred to sealed glass tubes and also saponified in alcoholic KOH. The remaining blood sample was centrifuged to sediment the blood cell elements, and aliquots of the plasma were then taken to determine the mass of water and the amount of tritium present per ml of plasma. From these data the specific activity of plasma water at the time the animal was killed could be calculated. Typically a specific activity of about 10 cpm/nmol of plasma water was achieved when 50 mCi of [^{3}H]water was given to a 200 g rat. The liver was weighed and aliquots in the range of 500 to 900 mg were placed in sealed glass tubes and saponified in alcoholic KOH. Following saponification of the three tissues, samples were taken in quadruplicate from the carcass, liver, and whole blood specimens, and used for extraction and isolation of the DPS as described below.

Determination of the specific activity of water in plasma and various organs

In one experiment the various organs were removed from the animals and cut into slices approximately 0.5 mm thick: these were then quickly blotted on filter paper and exact aliquots were weighed on an electric balance. One set of aliquots (usually three) was then dried overnight for determination of water content, while a second set of weighed aliquots was placed in sealed volumetric flasks for determination of ^{3}H content. Plasma was processed in similar fashion: weighed aliquots were taken to dryness to determine water content while other aliquots were placed in volumetric flasks for assaying the amount of ^{3}H. From the mass of water and the ^{3}H content in the aliquots of plasma and the different organs, the specific activity of water in the various tissues could be calculated.

Determination of synthesis rates in vitro

In another experiment, rates of hepatic sterol synthesis also were measured in vitro. Following the injection of [^{3}H]water and removal of the liver 1 hr later, as described above, aliquots of the liver were taken to determine rates of incorporation of [^{3}H]water into DPS in vivo. Other aliquots of the same liver were used to prepare liver slices on a mechanical tissue slicer as previously described (8). Three hundred mg-aliquots of these slices were then incubated in vitro with 1.0 mM octanoate as previously described, except that 10 μCi, rather than 1 μCi, of [1-^{14}C]octanoate was added to each incubation flask (8, 9). At the end of the incubation period, the rates of incorporation of [1-^{14}C]-octanoate into DPS and ketones were determined (8, 9, 30). Thus, in this experiment it was possible to determine rates of sterol synthesis in the same liver under both in vivo and in vitro conditions.

Isolation and purification of the radiolabeled sterols

Because we have previously shown that there is considerable carryover of [^{3}H]water during isolation of the sterol digitonides, a new procedure was utilized in isolating the radiolabeled sterols (10). After saponification, each of the samples was adjusted with water to contain an approximately 50% ethanol solution. The sterols were then quantitatively extracted using petroleum ether and precipitated as the digitonides as previously described in detail (14). The digitonin precipitates were washed twice with acetone and once with diethyl ether and were then taken to dryness under air. The precipitates were further dried for 15 min under vacuum at 80°C. The digitonin precipitates were then dissolved in pyridine and the free sterols were quantitatively extracted with diethyl ether and

taken to dryness under air (31). The dried, free sterol extracts were then placed in a vacuum oven at 80°C for an additional 1 hr; scintillation solution was then added to each vial and the samples were assayed for their ^{3}H or ^{14}C content.

Calculations

Several types of calculations were carried out in order to determine rates of sterol synthesis in the liver and carcass of the animals and to determine the amounts of ^{3}H-labeled DPS present in the circulating whole blood volume at the time the animals were killed. The weight of the whole animal and of the liver was determined directly when each rat was killed. The weight of the total blood volume was calculated from the weight of each animal using the mean density of rat blood and assuming that there was 6.4 ml of blood per 100 g body weight (32). The weight of the carcass was calculated by subtracting the weights of the liver and the 5.0 ml blood sample from the whole animal weight. The volume of blood remaining in the carcass at the time the assays were carried out, i.e., the residual blood volume, was determined by subtracting the 5.0 ml volume of blood aspirated from the aorta from the calculated total blood volume of the animal.

The ^{3}H-labeled DPS present in the samples of whole blood and liver could be determined directly: however, the radiolabeled sterols present in the carcass had to be corrected for the ^{3}H-labeled DPS present in the residual blood volume that was necessarily also processed with the other tissues of the carcass. This was accomplished by subtracting the product of the residual blood volume and the concentration of ^{3}H-labeled DPS per ml of whole blood from the total radioactivity in the carcass. This correction, it should be emphasized, was a small one and in most cases equaled $<10\%$ of the total radioactivity found in the carcass DPS.

The specific activity of plasma water was calculated using the following relationship:

$$\frac{(\text{cpm } ^{3}H/\text{ml plasma})(1.09)}{(\text{nmol water/ml water})(0.92 \text{ ml water/ml plasma})}$$

The term 1.09 corrects the specific activity of plasma water determined at 1 hr after injection of [^{3}H]water to the mean specific activity of body water present throughout the 1 hr period of time (see Fig. 2 and Results section). The term 0.92 corrects the calculation for the concentration of water determined in these studies to be present in 1.0 ml of plasma. From these two sets of data one can calculate the rates of incorporation of [^{3}H]water into DPS per hour per g of liver and carcass (nmol of [^{3}H]water/hr per g) or per ml of whole blood (nmol of [^{3}H]water/hr per ml).

In the studies in which rates of sterol synthesis were measured in liver slices in vitro, the data were calculated as the nmol of [1-^{14}C]octanoate incorporated into DPS per g of tissue per hour (nmol/g per hr). These rates were then corrected for dilution of the specific activity of the intracellular [1-^{14}C]acetyl CoA pool and converted to nmol of acetyl CoA units, i.e., C_2 units (8, 9). Thus, the absolute rates of sterol synthesis from [1-^{14}C]octanoate in vitro are given as the nmol of C_2 units incorporated into DPS per g per hour (nmol/g per hr). The rate of incorporation of [^{3}H]-water into DPS by the same liver in vivo was converted into the same units by multiplying this rate by the factor 1.45 which gives the μg atoms of carbon incorporated into DPS per μg atom of hydrogen (10).

In all cases, data are presented as the mean values $\pm$ 1 SEM for the number of animals used in each experiment. In several figures, linear regression curves were fitted to individual data points by the method of least squares.

RESULTS

The initial group of studies was undertaken to validate the techniques employed in these experiments to isolate and assay the amount of ^{3}H-labeled sterols synthesized in animals administered large amounts of [^{3}H]water. In a previous publication we have shown that when cholesterol is precipitated as the digitonide in the presence of [^{3}H]water, about 20 nmol of water is tightly bound to the precipitates for each 1.0 mg of cholesterol isolated as the digitonide (10). This contamination with [^{3}H]water cannot be eliminated by either heating the precipitates under vacuum or by repeated washing with acetone (10). However, we found that this source of error could be entirely eliminated if the free sterols were regenerated from the digitonides and carefully dried under vacuum. Thus, as outlined in detail in the Methods section, after the administration of [^{3}H]water to the experimental animals, the various tissues were saponified and the sterols were isolated as the digitonides. The digitonides were then dried under vacuum and split with pyridine, and the free sterols were extracted in diethyl ether. The ether extracts were then taken to dryness under air, heated at 80°C under vacuum, and assayed for [^{3}H]content.

It is critically important that this procedure be demonstrated to give essentially no contamination with [^{3}H]-water but essentially complete recovery of the radiolabeled sterols. In order to examine both of these prerequisites, the two studies illustrated in **Fig. 1** were

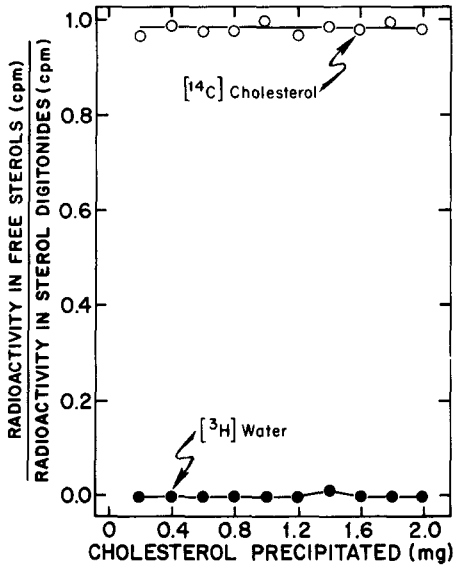

Fig. 1. Evaluation of [^{3}H]water carryover and [^{14}C]cholesterol recovery during the digitonin precipitation and regeneration of free cholesterol. This diagram shows results of two different types of experiments. In the first (lower curve), digitonin precipitation of 0.2 to 2.0 mg of unlabeled cholesterol was carried out in the presence of 1 mCi of [^{3}H]water. In the second experiment (upper curve), 1 μCi [^{14}C]cholesterol was precipitated with digitonin in the presence of 0.2 to 2.0 mg of unlabeled cholesterol. In both experiments the digitonin precipitates were then dried, split with pyridine, and again dried, as described in detail in the Materials and Methods Section. Aliquots were taken for assay of the radioactivity in the sterol digitonides and in the regenerated free sterols. The vertical axis gives the ratio of these two values found in each sample. Each point represents the mean of three separate experiments.

undertaken. In one experiment (closed circles) 0.2 to 2.0 mg of cholesterol was precipitated as the digitonide in the presence of 1 mCi of [^{3}H]water (in a volume of 1.0 ml of water). After the precipitates were split and dried, however, no radioactivity above the background levels could be detected in any of the samples, regardless of the amount of cholesterol precipitated (lower curve, Fig. 1). In a second experiment, authentic [^{14}C]-cholesterol was precipitated as the digitonide in the presence of 0.2 to 2.0 mg of unlabeled cholesterol. The sterol digitonides were dissolved in methanol and an aliquot was taken to determine the amount of [^{14}C] in the sample. A second aliquot was then taken to dryness, the sterol digitonides were split with pyridine, and the free sterols were extracted and dried. As also shown in Fig. 1 (open circles) >98% of the [^{14}C]cholesterol present in the sterol digitonides could be recovered in the free sterol preparations. Thus, we concluded from these two preliminary experiments that the method employed in these studies for isolation of radiolabeled tissue sterols essentially eliminates the problem of contamination of the sterols with [^{3}H]water and results in nearly complete recovery of authentic, radiolabeled cholesterol.

In order to calculate valid rate constants for sterol synthesis from [^{3}H]water, it is necessary to have an accurate measure of the specific activity of tissue water in each organ system throughout the interval of time during which rates of synthesis are being measured. A second group of studies was undertaken, therefore, to define the behavior of this parameter in the experimental animals at different times after the administration of [^{3}H]water. As shown in **Fig. 2,** after the essentially instantaneous intravenous administration of a bolus [^{3}H]water at time 0, the plasma water relative specific activity fell continuously between 2 and 45 min, but achieved a constant value between 45 and 120 min.

While a portion of the fall in the specific activity of plasma water during the early time points is undoubtedly due to mixing in the vascular compartment and to equilibration of [^{3}H]water between the intra-

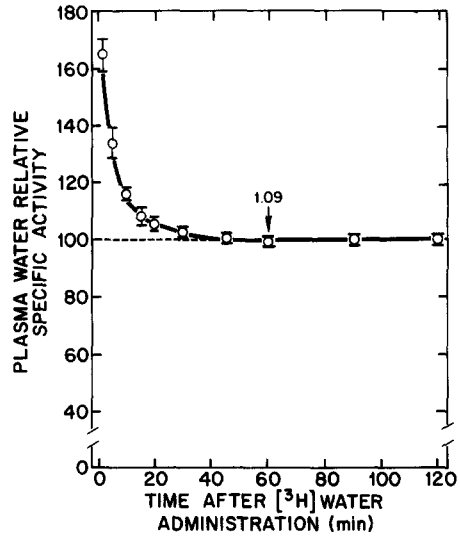

Fig. 2. Relative specific activity of plasma water as a function of time after the intravenous administration of [^{3}H]water to the experimental animals. Each rat was fitted with both a femoral vein and tail vein catheter and then placed in an individual restraining cage. At time 0 the animals were administered a dose of [^{3}H]water through the tail vein, following which the catheter was rapidly removed: this whole process required about 10 seconds. Blood from the inferior vena cava was then sampled through the femoral vein catheter at intervals varying from 2 to 120 minutes after the administration of the [^{3}H]water. A volume of blood equal to the dead space in the catheter was removed and discarded prior to obtaining each blood sample. All animals were sampled at 2 and 60 min, but the other sampling times were staggered in different animals to avoid removing more than a total of 1 ml of whole blood from any one animal. The blood was centrifuged and the plasma radioactivity was then assayed. The absolute specific activity of plasma at each time was divided by the specific activity found at 60 min in the same animal, and this ratio was multiplied by 100: thus, in this figure the plasma specific activity equaled 100 at 60 min and the values at the other sampling times are given relative to this value. This curve was constructed from data obtained when samples were taken from 12 different animals: each point represents the mean ± 1 SEM for four to six values at each time point except those obtained at 2 and 60 min which were derived from 12 data points.

vascular and other extracellular spaces, the continued more prolonged decline must reflect slower equilibration of the [^{3}H]water between the serum and intracellular water in the various organs, and/or bulk water present in such compartments as the urinary tract, biliary system, central nervous system, and gut. In order to determine which of these two compartments was predominantly responsible for the relatively slow equilibration of serum water during the first 45 min of these experiments, rats were administered [^{3}H]water intravenously and groups of animals were then killed at intervals and multiple organs removed. The specific activity of the tissue water in each organ was determined and was compared to the specific activity of the plasma water measured in that same animal. As shown in the case of the liver, intestine, kidney, and spleen in **Fig. 3,** the specific activity of tissue water equaled that of the plasma at all times, including the earliest time that a tissue was sampled, i.e., 5 min after the administration of [^{3}H]water. Similar findings were obtained in other major tissues of the body including the brain, lung, skeleton muscle, heart, adrenal gland, stomach, and colon. In contrast, in samples of bladder urine there was incomplete equilibration during the first 30 min of the experiment. Presumably, therefore, a portion of the fall in the specific activity of plasma water during the early time points (Fig. 2) reflects continued exchange with bulk water in the bladder and, probably also, in the central nervous system, gut contents, and biliary tract.

We concluded from this second group of experiments, however, that the specific activity of intracellular water equals the specific activity of plasma water at essentially all times after the administration of [^{3}H]-water intravenously. Thus, the specific activity of plasma water can be appropriately utilized to calculate rates of incorporation of [^{3}H]water into DPS. However, since the specific activity of plasma water falls somewhat during the initial 45 min of these studies (Fig. 2), a correction must be applied to these calculations in order to obtain an integrated value for the specific activity of intracellular water during the period of observation. For example, as shown in Fig. 2, in animals killed 1 hr after the injection of [^{3}H]water, the specific activity of the plasma water must be multiplied by a factor of 1.09 to give the mean specific activity of tissue water over the 60 min interval of the experiment. Different factors were established from these curves for animals killed at other time intervals.

With these factors established, it was next possible to examine the relationship between the amount of [^{3}H]water incorporated into DPS in different tissues and the time interval during which body water was labeled with ^{3}H. As shown in **Fig. 4,** in 20 rats injected

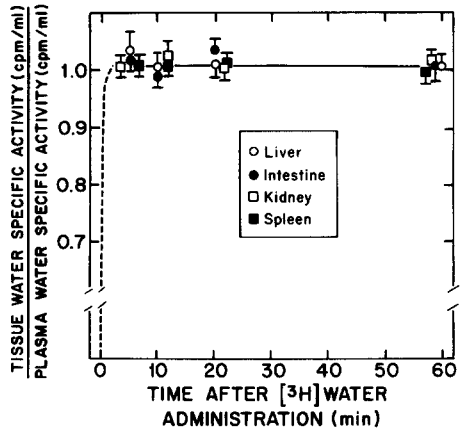

Fig. 3. Tissue water specific activity relative to plasma water specific activity at different times after administration of [^{3}H]water to the experimental animals. At time 0 the rats were given a dose of [^{3}H]water intravenously and groups of animals were then killed 5, 10, 20, and 60 min later. Various organs were immediately removed and assayed for content of water and [^{3}H]water. In this diagram the absolute specific activity of the tissue water found in liver intestine, kidney, and spleen was divided by the absolute specific activity of plasma water determined in the same animal. Each point represents the mean ± 1 SEM for four animals killed at each time point.

with [^{3}H]water at time 0, there was an apparent linear relationship between the amount of newly synthesized DPS found in the liver and the length of time, up to 60 min, after administration of the isotope. It should be noted that not only does this curve appear to extrapolate to an incorporation rate of 0 nmol/g at 0 time, but also in the five animals actually killed immediately after injection of the [^{3}H]water there was no detectable radioactivity in the DPS. This finding further supports the

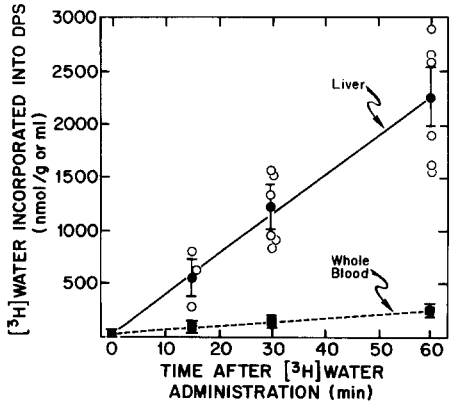

Fig. 4. Time course for the incorporation of [^{3}H]water into DPS in the liver and blood. At time 0 a group of 20 rats was administered [^{3}H]water intravenously and animals were then killed 15, 30, and 60 minutes later. Aliquots of liver and blood were obtained and assayed for their content ^{3}H-labeled DPS. The results are expressed as the nmol of [^{3}H]water incorporated into DPS per g of liver or per ml of whole blood. Results in individual animals are shown as open circles, while the closed circles and squares give the mean values ± 1 SEM for the animals killed at each time point.

validity of the technical procedures adopted in this study for the isolation of DPS in the presence of large amounts of [^{3}H]water. During this same time interval, ^{3}H-labeled DPS also appeared in whole blood (dashed line in Fig. 4) and in the remaining tissues of the carcass (not shown in Fig. 4) at rates that were apparently linear with respect to time after administration of the [^{3}H]water. Utilizing thin-layer chromatography it was found that at 60 min >94% of the ^{3}H present in the DPS of liver and blood co-chromatographed with authentic cholesterol while in the carcass this figure averaged about 82%.

On the basis of three considerations, we concluded that in all subsequent studies rates of DPS synthesis should be assessed 1 hr after administration of the [^{3}H]water. These considerations included the facts that *1*) incorporation rates are linear with respect to time during this interval, *2*) manipulation and restraint of rats for 1–2 hours does not alter rates of sterol synthesis in any tissue, and *3*) we wanted to minimize the time available for net movement of sterols from their sites of synthesis to other tissue compartments.

Utilizing this protocol, the next major group of experiments was undertaken to measure rates of sterol synthesis in vivo in the major tissue compartments of animals subjected to a variety of physiological manipulations. The results of these studies are summarized in **Table 1.** In control animals killed at the mid-dark phase of the light cycle (experimental group A), 2290 nmol/hr per g of [^{3}H]water was incorporated into DPS by the liver. Both cholesterol feeding and fasting markedly suppressed the amount of ^{3}H-labeled DPS present in the liver, to 103 and 271 nmol/hr per g, respectively, (groups B and C), while cholestyramine feeding increased the apparent rate of hepatic sterol synthesis over twofold to 4800 nmol/hr/g (group E). When the fasted animals were subjected to the stress of being placed in restraining cages for 48 hr, hepatic DPS synthesis increased nearly five-fold to 1290 nmol/hr per g (group D versus group C). In the two matched groups of animals subjected to oppositely phased light cycles (groups F and G) there was a clear diurnal rhythm in hepatic sterol synthesis: the rates equaled 532 and 1150 nmol/hr per g, respectively, at the mid-light and mid-dark point of the light cycle. As is also evident in Table 1, there was generally a correlation between the apparent rate of hepatic sterol synthesis and the amount of ^{3}H-labeled DPS that was present in whole blood 1 hr after administration of the [^{3}H]-water (column 5). In contrast, in most experimental groups, the apparent rate of sterol synthesis in the carcass correlated poorly or not at all with the rate found in the liver. For example, cholesterol feeding suppressed hepatic synthesis to only 4% of the control level, but resulted in virtually no suppression of the apparent rate of synthesis in the carcass (group B versus A). When fasted animals were stressed, hepatic synthesis increased 476% while the rate of synthesis in the extrahepatic tissues changed by only 35% (group D versus C). In the animals subjected to light cycling, there was no difference in the apparent rate of carcass synthesis even though there was a definite diurnal variation in hepatic synthesis (group F versus G).

As shown in the last four columns of Table 1, these data can also be presented in terms of apparent rates of sterol synthesis in the whole liver and in the remaining tissues of the carcass. In control animals (group A) whole body sterol synthesis was such that 34.5 μmol of [^{3}H]water was incorporated into DPS per hour: of this amount only 46.5% was found in the liver, 7.7% was in the circulating blood, and 45.8% was in the remaining organ systems of the carcass. Cholesterol feeding and fasting suppressed whole body sterol synthesis to 14.04 and 9.39 μmol/hr, respectively, and in both cases >80% of this synthesis was accounted for by incorporation of [^{3}H]water into DPS in the extrahepatic tissues of the carcass. Even in control animals subjected to light cycling, sterol synthesis in the tissues of the carcass accounted for nearly 70% of whole body sterol synthesis when the nadir of hepatic synthesis was reached during the mid-light point of the light cycle. These data once again emphasize the responsiveness of the liver to the various physiological manipulations tested in this study: the apparent contribution of liver to whole body synthesis varies from a low of only 4.5% in cholesterol feeding to 57.5% when the enterohepatic circulation of bile acids is interrupted by cholestyramine feeding.

A major problem with these latter studies is that there is a possibility that DPS might be synthesized in one tissue site and then rapidly transported to a second tissue, even within the relatively short period of time during which these experiments were performed. If such an event occurred, it might lead to errors in determining the tissue distribution of sterol synthetic sites. In order to examine this important possibility more fully, two additional types of experiments were undertaken. In the first study, 36 animals were subjected to a variety of manipulations including cholesterol and cholestyramine feeding, fasting, and stress. In addition, control animals of different size were utilized at all points during the light cycle. The purpose of these manipulations was to obtain a group of animals with apparent rates of hepatic sterol synthesis that varied over a 1000-fold range. Each of these animals were then administered [^{3}H]water, and the content of ^{3}H-labeled DPS in liver, whole blood and carcass was assayed as in those experiments shown in Table 1. In

TABLE 1. In vivo rates of sterol synthesis in the liver and carcass of animals subjected to various physiological manipulations

Experimental[a] Group	Weights			[^{3}H]Water Incorporation Into DPS Per Unit Tissue[b]			[^{3}H]Water Incorporation Into DPS Per Whole Tissue[c]			
	1 Whole Animal	2 Liver	3 Carcass	4 Liver	5 Blood	6 Carcass	7 Liver	8 Blood	9 Carcass	10 Whole Animal
	g			*nmol/hr/g or ml*			*μmol/hr/whole tissue*			
A. Control	172 ± 9	7.2 ± 0.4	161 ± 9	2,290 ± 268	247 ± 28	103 ± 15	16.10 ± 1.72 (46.5%)	2.59 ± 9.19 (7.7%)	15.80 ± 1.51 (45.8%)	34.50 ± 2.87
B. Cholesterol fed	178 ± 11	7.5 ± 0.5	166 ± 11	103 ± 38	66 ± 10	81 ± 15	0.71 ± 0.24 (4.5%)	0.73 ± 0.12 (5.7%)	12.59 ± 1.58 (89.8%)	14.04 ± 1.76
C. Fasted	154 ± 4	5.1 ± 0.3	143 ± 4	271 ± 66	35 ± 6	54 ± 2	1.41 ± 0.37 (14.5%)	0.34 ± 0.06 (3.6%)	7.64 ± 0.07 (81.9%)	9.39 ± 0.43
D. Fasted, stressed	136 ± 4	5.1 ± 0.4	126 ± 3	1,290 ± 110	118 ± 15	73 ± 3	6.69 ± 0.84 (39.4%)	0.87 ± 0.15 (5.2%)	9.21 ± 0.60 (55.4%)	16.78 ± 1.36
E. Cholestyramine fed	183 ± 5	7.3 ± 0.2	171 ± 5	4,800 ± 599	479 ± 39	121 ± 16	35.12 ± 3.8 (57.5%)	5.25 ± 0.48 (8.3%)	20.53 ± 2.28 (34.2%)	60.90 ± 5.58
F. Mid-dark	203 ± 5	7.3 ± 0.6	190 ± 5	1,150 ± 165	117 ± 20	54 ± 5	8.46 ± 1.37 (41.9%)	1.47 ± 0.23 (7.2%)	10.25 ± 0.69 (50.9%)	20.18 ± 2.62
G. Mid-light	209 ± 9	7.2 ± 0.7	197 ± 8	532 ± 39	60 ± 4	52 ± 6	3.80 ± 0.30 (25.8%)	0.79 ± 0.08 (5.4%)	10.23 ± 0.89 (68.8%)	14.82 ± 0.96

[a] In groups A–E, animals were subjected to light cycling for 2 weeks and were then either fed 2% cholesterol or 3% cholestyramine for 4 days, fasted for 48 hr or fasted and stressed for 48 hr before rates of sterol synthesis were measured in vivo at the mid-dark point of the light cycle. The animals in groups F and G were from the same shipment of rats and were placed in two light-cycling chambers for 3 weeks before being used in this experiment. The cycle of 12 hr of light and 12 hr of darkness was reversed in the two chambers so that when rates of sterol synthesis were measured at 0900 hr, half of the animals were at the mid-dark point while the others were at the mid-light point of the light cycle. All values in this table represent means ± 1 SEM for data obtained with six animals in each group.

[b] Expressed as the nmol of [^{3}H]water found in the DPS in 1 ml of whole blood or in 1 g of liver or carcass 1 hr after administration of the [^{3}H]water.

[c] The volume of whole blood was assumed to equal 6.4 ml per 100 g body weight. The percentage values give the fraction of whole animal sterol synthesis (column 10) found in each tissue compartment 1 hr after administration of the [^{3}H]water.

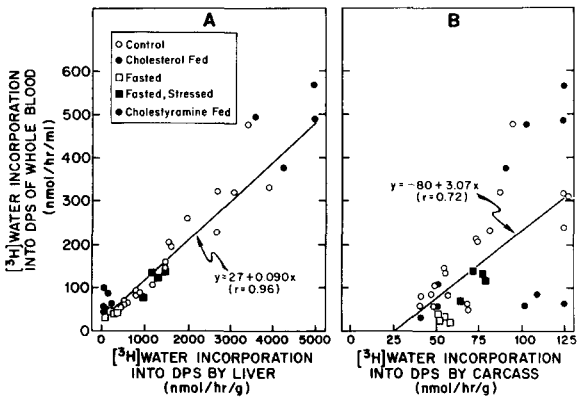

Fig. 5. Relationship between the amount of ^{3}H-labeled DPS present in blood and the rates of sterol synthesis in the liver and carcass. The animals used in this study were subjected to a variety of physiological manipulations as listed in the insert. It should be noted that the animals designated as control rats varied in size (150–227 g) and were killed at all times during the light cycle in order to obtain a wide range of rates of synthesis in normal animals. Each animal was administered [^{3}H]water and killed 1 hr later. The amount of ^{3}H-labeled DPS present in the blood was assayed (vertical axis) and is plotted in this figure against the amount of ^{3}H-labeled DPS present at the same time in the liver (Panel A) or in the extrahepatic tissues of the carcass (Panel B). The points represent the results obtained in each individual animal.

panel A of **Fig. 5,** the amount of ^{3}H-labeled DPS that appeared in 1 hr in the whole blood of each animal is plotted against the rate of sterol synthesis in the liver of that same rat. As is apparent there is an excellent correlation (r = 0.96) between the apparent rate of hepatic synthesis and the amount of ^{3}H-labeled DPS appearing in blood. Furthermore, the linear regression curve extrapolates nearly to 0 nmol/hr per ml as synthesis in the liver approaches 0 nmol/hr per g. In contrast, when the amount of ^{3}H-labeled DPS in blood is plotted against the apparent rate of DPS synthesis in carcass (panel B), the correlation is much poorer and there are several groups of animals in which there is complete dissociation between the rate of sterol synthesis in the carcass and the amount of [^{3}H]DPS appearing in blood.

In a second study, five rats were subjected to partial hepatectomy just prior to administration of the [^{3}H]-water. In five sham-operated, control animals the rate of [^{3}H]water incorporation into DPS in whole liver, blood, and carcass equalled 17.1, 2.9, and 18.7 μmol/hr. In the animals in which, on average, 68% of the liver had been acutely removed, these three rates equaled 4.7, 0.9, and 19.6 μmol/hr, respectively. Thus, the amount of ^{3}H-labeled DPS present in liver and blood decreased in proportion to the amount of liver removed, while the rate of synthesis in the tissues of the carcass was essentially unaffected by partial hepatectomy. These results, along with those shown in Fig. 5, provide strong, although indirect, evidence that the ^{3}H-labeled DPS found in blood under the conditions of these experiments is essentially all synthesized in the liver while the ^{3}H-labeled DPS found in the carcass is synthesized locally within those tissues.

In a final set of studies, shown in **Fig. 6,** a direct comparison was made between the rates of hepatic sterol synthesis measured under both in vivo and in vitro conditions in the same animal. In order to obtain a range of synthetic rates, the experimental animals were subjected to the physiological manipulations shown in the insert to Fig. 6. Each animal was administered [^{3}H]water and killed 1 hr later. Aliquots of liver and blood were obtained to assay ^{3}H-labeled DPS content, and other aliquots of the liver were used to prepare liver slices that were then incubated with [1-^{14}C]octanoate in vitro. The absolute rates of acetyl CoA, i.e., C_2 units, incorporated into DPS under these in vitro conditions was calculated after correcting for intracellular dilution of the specific activity of the acetyl CoA pool: these data are plotted on the horizontal axis. The absolute rates of acetyl CoA incorporated into DPS under in vivo conditions was calculated from the rates of incorporation of [^{3}H]water assuming that 1.45 μg atoms of carbon are incorporated into the sterol molecule for each μg atom of hydrogen (10): these data are plotted on the vertical axis. It is apparent that there is an excellent correlation (r = 0.98) between the rates of hepatic sterol synthesis measured both in vivo and in vitro over a range of values that varied nearly 1000-fold. However, as is also evident the ab-

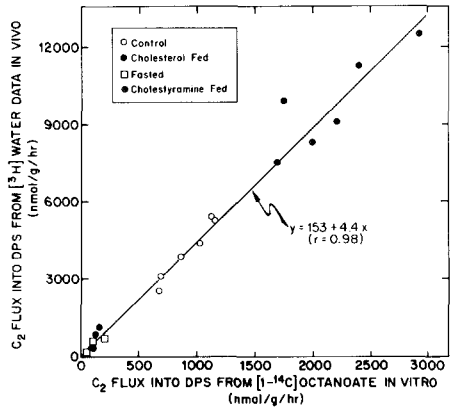

Fig. 6. Relationship between the rate of hepatic sterol synthesis measured in the same liver under both in vivo and in vitro conditions. The animals used in this experiment were subjected to the various physiological manipulations shown in the insert. Each rat was then administered [^{3}H]water and killed 1 hr later. Aliquots of liver were taken for determination of ^{3}H-labeled DPS content and, in addition, liver slices were prepared and incubated in vitro with [1-^{14}C]octanoate. By appropriate calculations, as described in the Materials and Methods Section, the measurements of rates of sterol synthesis under both in vivo (vertical axis) and in vitro (horizontal axis) conditions were normalized to the same units, i.e., the nmol of acetyl-CoA (C_2) units incorporated into DPS per g of liver per hour.

solute rates of sterol synthesis measured in vivo averaged 4.4-fold higher than the rates measured in the same livers under in vitro conditions. Presumably, this situation is attributable to damage to a significant number of hepatocytes during preparation of the slices, or to such factors as oxygen deficit, or changes in optimum concentrations of cofactors within the cells during the period of incubation. Thus, the rates of sterol synthesis measured in vitro give correct *relative* changes in synthetic activity, but these values are in error with respect to the *absolute* magnitude of these rates of DPS synthesis.

DISCUSSION

Despite rather extensive investigations into the mechanisms of regulation of cholesterol metabolism, there has been little success in directly comparing absolute rates of sterol synthesis, determined with various radiolabeled precursors in different organs or in the whole animal or human subject, and rates of synthesis determined by external sterol balance techniques. The inability to obtain comparable data by these types of measurements derives primarily from several major technical problems related to the use of enzyme assays or the incorporation of radiolabeled precursors into sterols as measures of absolute rates of sterol synthesis under either in vitro or in vivo conditions. For example, one commonly utilized method for assaying rates of cholesterol synthesis involves measurement of the activity of microsomal HMG CoA reductase activity. However, recent publications from several laboratories have shown that the activity of this enzyme is altered as much as 10-fold by phosphorylation-dephosphorylation reactions that apparently can take place during homogenization of the tissue and preparation of the microsomes (6, 7). It is not clear at this time whether it is the fully phosphorylated (inactive) or fully dephosphorylated (active) preparations that reflect the true rates of enzyme activity that exist within the cell in vivo. It is nearly impossible, therefore, to extrapolate from such enzyme activity measurements to absolute rates of sterol synthesis in the intact animal.

A second commonly utilized method for assaying the level of sterol synthesis is to measure the rate of incorporation of a ^{14}C-labeled substrate like acetate, glucose, pyruvate, or octanoate into cholesterol by a whole cell preparation such as tissue slices or organ biopsies. It is now recognized that such a method commonly yields rates that are underestimates of the true rates of synthesis. This underestimation occurs for at least three reasons: *1*) the rate of penetration of the cell membranes by the potential substrate may be rate limiting; *2*) the rate of metabolism of the substrate to acetyl CoA may be rate limiting; or *3*) the specific activity of the substrate or of the acetyl CoA generated from the substrate may undergo dilution within the cell (10). These limitations, it should be emphasized, may produce even greater artifacts if an attempt is made to use such ^{14}C-labeled substrates to measure rates of synthesis in vivo. In a few instances it has been possible to circumvent all three of these difficulties by choosing a substrate that penetrates cell membranes and is oxidized to acetate CoA at very high rates, and then correcting mathematically for any residual dilution of the specific activity of the intracellular acetyl CoA pool. Such conditions are met, for example, when [1-^{14}C]octanoate incorporation into DPS is used as a measure of the rate of sterol synthesis in liver slices (8, 9, 22, 30). In the majority of tissues, however, it has not been possible to establish these conditions so that rates of sterol synthesis measured with such substrates as [^{14}C]acetate or [^{14}C]glucose may equal only 2% to 40% of the true rates of sterol synthesis occurring in tissues like liver, skin, muscle, lung, and various endocrine glands (10).

A third problem with in vitro assay systems is that there may be nonspecific damage during preparation of the tissue slices or isolated cells. Furthermore, during the period of incubation there may be alterations in cell function that result from such factors as poor oxygenation or a change in cofactor concentrations within the cytosolic compartment. Whatever the nature of these changes, it is clear that rates of synthesis measured in tissue slices are always significantly lower than those measured in the anatomically intact organ. For example, the rate of incorporation of [^{3}H]water into DPS by liver slices from control animals in vitro equals approximately 328 to 521 nmol/hr per g (10) while rates of incorporation of nearly 2,300 nmols/hr per g were found in the present study in vivo (Table 1). Similarly high rates of [^{3}H]water incorporation into DPS have been found in the isolated perfused liver (33, 34). This marked discrepancy is further emphasized by the studies shown in Fig. 6, where absolute rates of synthesis were measured both in vivo and in vitro in the same liver, utilizing techniques that circumvent the problems of cell penetration and intracellular dilution of the specific activity of the acetyl CoA pool. Over a very wide range of synthetic activity, the in vitro assay system gave rates of acetyl CoA incorporation into DPS that were only about 23% of those rates found in vivo in the same animal.

Despite these major problems, it should be emphasized that measurement of HMG CoA reductase activity or rates of incorporation of various [^{14}C]substrates

into DPS still are useful ways in which to follow *relative* changes in rates of sterol synthesis in a given tissue under different physiological circumstances. It must be recognized, however, that such measurements may not reflect the true rates of synthesis occurring in that tissue under in vivo conditions and may not yield data on which to make a valid comparison of rates of synthesis between different tissues.

The use of [^{3}H]water for measuring rates of cholesterol synthesis overcomes the three major problems associated with the use of ^{14}C-labeled substrates. The membranes of most tissues are very permeable to water so that the specific activity of cell water rapidly equilibrates with the specific activity of extracellular water: this point is illustrated by the results of the in vivo studies illustrated in Fig. 3. [^{3}H]water does not require extensive metabolism prior to incorporation into sterol molecule. The ^{3}H atom is incorporated into the cholesterol molecule either directly from [^{3}H]water or after equilibration with the reductive H of NADPH (34). Finally, the rate of metabolic generation of unlabeled water is extremely small relative to the very large mass of water present in either the in vitro or in vivo systems. There is virtually no possibility, therefore, that there will be any detectable dilution of the specific activity of [^{3}H]water during the course of the experiment. It should also be noted that use of [^{3}H]water will not distort the size or distribution of the precursor pool of acetyl CoA in the cell as clearly occurs when large amounts of a [^{14}C]substrate are added to the system (8–10).

The one major theoretical problem associated with the use of [^{3}H]water is that absolute rates of cholesterol synthesis can be calculated from the rates of [^{3}H]water incorporation into DPS only if one has a reliable value delineating the number of ^{3}H atoms that are incorporated into each molecule of cholesterol. Furthermore, it must be determined that this ratio is constant in a given tissue under many physiological circumstances and that the ratio is the same in the cells of different organ systems. While data on these points are limited, it has been shown that in liver, both in slices and in the isolated perfused organ, approximately 0.69 μg atoms of ^{3}H are incorporated from [^{3}H]water into DPS for each μg atom of C that enters the cholesterol biosynthetic pathway as acetyl CoA (10, 33). Furthermore, this value is the same under circumstances where the liver was obtained from animals subjected to such manipulations as fasting or cholestyramine feeding (10). This ratio has not been measured in extrahepatic tissues: however, in rats subjected to long term administration of either [^{3}H]water or deuterium-labeled water, the "whole-body" incorporation ratio equaled 0.69 and 0.64, respectively (35, 36). Since the extrahepatic tissues make a significant contribution to total body synthesis, it follows from these two observations that the ^{3}H/C incorporation ratio in the majority of these tissues must essentially equal the value found in the liver. Nevertheless, it must be emphasized that the value of this ratio could conceivably deviate significantly from 0.69 in an organ system that contributed only a small amount of sterol to the whole body pool of newly synthesized cholesterol.

With this reservation in mind, four major conclusions of physiological importance can be derived from these studies. The first conclusion deals with the identification of control mechanisms that operate in the various organ systems under in vivo conditions. As shown in Table 1, for example, there is a definite diurnal variation in hepatic sterol synthesis, as has been previously reported (27, 28), and the level of incorporation of [^{3}H]water into DPS is markedly suppressed by cholesterol feeding or fasting, and is significantly enhanced by stress or by cholestyramine feeding. In addition, an increase in the size of the animals from 172 to 203 g (groups A versus F) also is associated with a fall in the rate of hepatic sterol synthesis. Essentially the same relative changes in rates of synthesis have been identified in rats of similar age using in vitro techniques that measure the absolute rates of acetyl CoA incorporation into sterols in liver slices (22). In contrast, the tissues of the carcass do not manifest a diurnal rhythm in synthetic activity, and the rates of [^{3}H]water incorporation into DPS are not dramatically changed by any of the other physiological manipulations. Only fasting modestly suppresses the mean rate of sterol synthesis seen in vivo in the tissues of the carcass. Again, these findings are similar to those reported in several detailed studies of the regulation of cholesterol synthesis in extrahepatic tissues utilizing the incorporation of [^{14}C]acetate into DPS as a measure of synthetic activity in tissue slices (15). We concluded from these observations that all of the major regulatory mechanisms that have been identified by in vitro techniques also operate to regulate cholesterol synthesis in vivo.

The second point derived from these studies relates to the relative importance of liver and the extrahepatic tissues in the carcass as quantitatively important sites for whole body sterol synthesis. In a recent analysis of the errors inherent in the use of ^{14}C-labeled substrates, we found that the degree of underestimation of true rates of sterol synthesis was much greater in extrahepatic tissues than in the liver and predicted from this result that in vivo, sterol synthesis in tissues of the body other than the liver would be found to be quantitatively far more important than previously reported (10). This prediction was substantiated in the

present study where, even in the control rats at the mid-dark point of the light cycle, nearly 46% of the sterol synthesis detected in the whole animal apparently took place in the extrahepatic tissues. The contribution of these tissues increased into the range of 70–90% of whole animal synthesis rates when the level of hepatic cholesterol synthesis fell during the mid-light phase of the diurnal cycle or after fasting or cholesterol feeding. Because of the responsiveness of sterol synthesis in the liver to these regulatory mechanisms, the contribution of this organ to whole animal sterol synthesis varied over a wide range, from a low of only 10% (^{3}H-labeled DPS in liver plus blood) to a high of 65%. As discussed above, the validity of these calculations is based on the assumption that the ^{3}H/C incorporation ratio in the tissues of the carcass is essentially the same as that in the liver. However, the value of the ratio of 0.69 corresponds to the situation where the reductive H of NADPH is fully equilibrated with [^{3}H]water (10, 34). If the ^{3}H/C incorporation ratio in carcass is different from that in liver, then it could only vary in the direction of being lower. Such a lower value would actually further increase, not diminish, the quantitative importance of sterol synthesis in the tissues of the carcass. Thus, the second important conclusion from these studies is that the various tissues of the carcass are quantitatively more important to total body sterol synthesis than previously recognized.

The data presented in column 10 of Table 1 also provide new information on the manner in which whole body sterol synthesis varies with these different manipulations. With cholesterol feeding and fasting, for example, total sterol synthesis in the rat is reduced to 41% and 27%, respectively, of the control values. Interruption of the enterohepatic circulation of bile acids, on the other hand, increases whole body sterol synthesis to 177% of control. These findings are very similar to changes in whole body sterol synthesis rates measured in man by external sterol balance techniques. Generally, cholesterol feeding or fasting is associated with a reduction in whole body sterol synthesis while interruption of cholesterol absorption or of the enterohepatic circulation of bile acids results in a marked increase in sterol synthesis in human subjects (37–40). Thus, the third conclusion derived from these experiments is that similar relative changes in whole body sterol synthesis were found with this isotope technique in the rat as have been demonstrated in man utilizing external balance procedures.

Finally, it is also possible to compare the quantity of total sterol synthesized in the rat as determined by both the isotope incorporation and external sterol balance techniques. The animals in groups F and G (Table 1) had an average weight of 206 g and a mean rate of [^{3}H]water incorporation into DPS of 17.5 μmol/hr. Since 1.45 μmol of acetyl CoA units are incorporated into sterols for each μmol of [^{3}H]water (the reciprocal of the ^{3}H/C incorporation ratio), and since 18 μmol of acetyl CoA are needed for the synthesis of 1 μmol of cholesterol, this corresponds to a rate of cholesterol synthesis of 1.41 μmol/hr or 33.8 μmol/day per animal (13.1 mg/day per animal). From a previous report, it can be calculated that in this size rat the fecal output of cholesterol, coprostanol, and taurocholate equals 8.4, 3.2, and 10.8 μmol/day, respectively, giving a total sterol output of 22.4 μmol/day (41). Given the facts that these external balance studies did not quantitate the output of sterol from the hair and skin (about 3.0 μmol/day) or as other bile acids (about 2.8 μmol/day), and that no attempt was made to correct for bacterial degradation of the sterol molecule in the intestine, these two values of whole body synthesis are in very close agreement. It is evident, however, that in the future these two types of measurements should be made in the same animals, as well as in several different species of animals, in order to validate the use of [^{3}H]-water as a relatively simple and rapid method for determining whole body sterol synthesis rates.

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