Chapter 1

QUANTITATIVE AND CONCEPTUAL CONTRIBUTIONS OF MATHEMATICAL MODELING TO CURRENT VIEWS ON VITAMIN A METABOLISM, BIOCHEMISTRY, AND NUTRITION

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I. INTRODUCTION

About 15 years ago, our colleague B. A. Underwood wondered whether the mathematical modeling methods we were applying to cholesterol metabolism might be used as a different approach to determine vitamin A utilization (i.e., disposal rate) and dietary requirements. Her interest, and our initial collaboration, led to subsequent research in our lab on vitamin A dynamics in the rat. Here we integrate information and ideas that have been generated by our application of mathematical modeling to vitamin A kinetic data as a way of illustrating how this approach has advanced and complicated our understanding of retinol metabolism. In most instances,

our paper does not review the many contributions of other investigators to this field. Rather our goal is to demonstrate that the application of a variety of modeling techniques to the vitamin A system has resulted in many different levels of insight into the metabolism, biochemistry, and nutrition of this fascinating essential nutrient.

II. HISTORICAL PERSPECTIVE AND EARLY STUDIES

Fifteen years ago, whole-body vitamin A metabolism was described as a rather simple process (for a review, see Goodman, 1980). It was known that the lipid-soluble vitamin was absorbed from the small intestine and that retinyl esters were packaged as a component of triglyceride-rich absorptive lipoproteins (chylomicrons) (Fig. 1). Triglyceride-depleted, vitamin A-containing chylomicron remnants were thought to be quantitatively cleared by the liver; there the vitamin was processed and secreted into plasma bound to its specific plasma transport protein, retinol-binding protein (RBP). In plasma, RBP binds to a larger protein, transthyretin (TTR), in a 1 : 1 molar ratio. After secretion from the liver, the retinolRBP complex was assumed to deliver retinol to vitamin A-dependent peripheral tissues where it was used for vitamin A action. Metabolites, possibly retinoic acid and other oxidized derivatives, were assumed to be excreted in both urine and feces. In addition to the liver, kidneys were believed to be an important organ in whole-body vitamin A metabolism.

After Underwood wondered in 1977 whether we could use kinetic methods to estimate vitamin A disposal rate in the rat, we collaborated on an *in vivo* kinetic experiment in vitamin A-deficient and control rats (Lewis *et al.,* 1981). We measured plasma [^{3}H]vitamin A disappearance for 48 hr after intravenous administration of [^{3}H]retinol-labeled plasma and used graphical methods to calculate vitamin A disposal rate. The results sug-

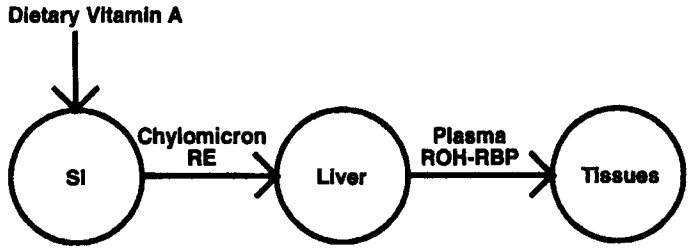

FIG. 1. Initial conceptual model for vitamin A metabolism. SI, small intestine; RE, retinyl esters; ROH, retinol; RBP, retinol-binding protein.

gested that vitamin A metabolism was much more complex than previously thought. Specifically, the fact that a semilogarithmic plot of the plasma response data did not follow a single exponential function indicated either retinol recycling to plasma or kinetic heterogeneity of plasma retinol. Furthermore, recovery of much of the label in the liver at 48 hr indicated that plasma retinol was recycled to liver. The calculated disposal rate in vitamin A-sufficient rats was many fold higher than expected, indicating that the tracer had not sufficiently mixed with the endogenous vitamin A pools. Thus we realized that a much longer experiment was needed to study vitamin A dynamics and to accurately estimate vitamin A disposal rate. As a consequence, we designed several large scale kinetic studies to characterize whole-body vitamin A metabolism in rats at different levels of vitamin A nutriture and we used model-based compartmental analysis (Green and Green, 1990a) to analyze the data. Before summarizing those results, we will briefly describe our experimental approach.

III. EXPERIMENTAL CONSIDERATIONS

A. EXPERIMENTAL DESIGN FOR *IN VIVO* STUDIES

See Green and Green (1990b) for further details. To date, our studies have all been done in adult male rats at one of several levels of vitamin A status. Rats have been used extensively in studies of vitamin A metabolism and are generally believed to be a good model for vitamin A metabolism in humans. In our experiments, rats to be used as recipients of labeled vitamin A are fed purified diets (Duncan *et al.*, 1993) containing various levels of vitamin A as retinyl palmitate to establish liver vitamin A reserves from deficient to high (<10 to >3500 nmol). It is ideal for rats to be in a steady state with respect to vitamin A during turnover studies, although the modeling programs used can accommodate nonsteady state situations. A physiological tracer of vitamin A (see next section) is prepared in donor rats and administered intravenously to recipients. Serial plasma and tissue samples are collected from ~10 min after injection until the end of the study (35115 days, depending on vitamin A status of recipients). Samples are extracted and analyzed for vitamin A radioactivity and, in some cases, vitamin A mass. Although care must be taken to ensure accuracy at each analytical step, it is worth emphasizing that reliable tracer data depends on adequate sample counting time. In our studies, all samples are counted twice to a 2-sigma error of <2.0% (i.e., to an accumulation of 10,000 counts per sample per counting cycle).

For kinetic analysis, data on sample radioactivity are converted to fraction of injected dose of [^{3}H]retinol vs time. Then individual rat data or group average values are analyzed using the Simulation, Analysis, and Modeling computer program (SAAM) (Berman and Weiss, 1978) and its conversational version (CONSAM) (Berman *et al.*, 1983). The SAAM program contains components dating back to 1959 and was developed by the late M. Berman and colleagues. It is continually being expanded and revised. Current versions of the program provide a powerful array of features for mathematical analysis of biological systems. See Foster and Boston (1983) for an excellent introduction to the use of SAAM/CONSAM. Distribution of the software, and excellent technical support and training are available from L. A. Zech (National Cancer Institute, NIH, Bethesda, MD) and D. M. Foster (Resource Facility for Kinetic Analysis, University of Washington, Seattle, WA) and their collaborators.

Choice of a compartmental modeling approach depends on the data available and the experimental aims. In our lab, we have used SAAM/CONSAM to analyze both *in vivo* and *in vitro* kinetic data by either model-based compartmental analysis or empirical compartmental analysis (Green and Green, 1990a). Working with *in vivo* data in the former case, a whole-body compartmental model is developed to fit all available data and other known characteristics of the system. For the more straightforward empirical compartmental analysis, plasma tracer disappearance data are fit to a multi-exponential equation, and identifiable kinetic parameters are calculated from the exponential constants and coefficients. In both cases, kinetic parameters, including fractional transfer coefficients, transit and residence times, transfer rates, disposal rates, and compartment masses are calculated using standard methods. For example, see Shipley and Clark (1972), Gurpide (1975), Jacquez (1985), and Green and Green (1990b).

Deciding how many samples to collect, and the optimal times for obtaining them, are important factors in the design of kinetic studies. Note that practical considerations (e.g., effect of frequent blood sampling on plasma volume, stress incurred by closely spaced blood samples, investigators' need for sleep, etc.) should be realistically evaluated and may sometimes compromise an optimal design. First, one needs to decide on the duration of the kinetic study. In the case of vitamin A, the length of experiments is influenced by the extent of liver vitamin A stores: since tracer must mix with slowly turning-over endogenous vitamin A, larger stores necessitate longer studies. Also investigators should conceptualize an initial model so that enough data are collected to ensure the best chances of parameter identification. Collecting three data points for each parameter expected will provide two degrees of freedom for each parameter being identified.

In our first long-term vitamin A kinetic study (Green *et al.,* 1985), we used a geometric progression to determine sampling times for plasma. For subsequent experiments, one can apply sensitivity analysis (Berman *et al.,* 1983) so that samples are collected at times significantly affecting the data that determine the kinetic parameters being identified. For example, it is critical to obtain data that will accurately define the terminal slope of a semilogarithmic plot of the plasma disappearance curve. Although the plasma response curve may seem uninteresting at that time, small changes in slope have a dramatic effect on parameter estimates. Specifically, the terminal slope is a function of the system fractional catabolic rate, which is an important parameter for calculating the disposal rate.

B. PHYSIOLOGICAL DOSES

In our studies, we have administered tritium-labeled vitamin A in one of its two physiological plasma transport vehicles (associated with either retinol-binding protein or chylomicrons) so that tracer data can be extrapolated to the vitamin A compounds of interest (retinol, retinyl esters, and metabolites). To prepare [^{3}H]retinol in its plasma transport complex (Green and Green, 1990b), vitamin A-depleted rats are used as donors to maximize hepatic secretion of the labeled vitamin on accumulated liver apoRBP. [^{3}H]Retinol or [^{3}H]retinyl acetate in an emulsion with Tween 40 is administered intravenously to donor rats and blood is harvested ~100 min later when plasma radioactivity is maximal. Plasma is isolated and stored under a nitrogen atmosphere at 4°C; plasma is used for *in vivo* studies within 23 days.

To prepare [^{3}H]vitamin A-labeled chylomicrons (Green *et al.,* 1993), [^{3}H]retinol or retinyl acetate is administered intraduodenally to thoracic lymph duct-cannulated donor rats and lymph is collected at 4°C. Chylomicrons containing mainly [^{3}H]retinyl esters can be isolated from lymph by preparative ultracentrifugation for administration to recipient rats. Alternatively, aliquots of whole lymph can be injected to minimize handling of the dose (see below). Then the proportions of total lymph radioactivity and vitamin A mass in chylomicrons (typically >8590%) can be determined analytically. In either case, lymph preparations should be used for *in vivo* studies within 12 days of collection.

Even when care is taken to handle the doses carefully, we have found that 215% of the tracer in the case of [^{3}H]retinol-labeled plasma, and up to 40% in the case of isolated [^{3}H]retinyl ester-labeled chylomicrons, acts "nonphysiologically" when preparations are injected into recipient rats. That is, a variable fraction of the dose (the "nonphysiological" component) is cleared from plasma within a few minutes. Presumably nonphysiological

components result from protein denaturation, chylomicron aggregation, or other physical changes caused by ultracentrifugation or by contact with air, glass, etc. In order to avoid these problems, we recommend that (1) maximal care, and minimal handling, be applied during preparation and administration of the labeled dose; (2) both a physiological and nonphysiological component of the dose be included during model development; and (3) an early plasma sample be collected (10 min or less after dose administration) so that the extent of the problem can be determined. It is not uncommon for investigators to normalize their data to the amount of tracer in the first plasma sample. Presentation of the data in this way merely masks the problem.

Although we believe that our *in vivo* methods produce physiologically relevant tracers of vitamin A, we and others are currently exploring alternative techniques for preparing high specific activity, physiological tracers for retinol and especially RBP. As is the case for the [^{3}H]retinol-labeled plasma and chylomicrons discussed above, such techniques are aimed at eliminating the need for protein iodination which may induce alterations in the protein that alter its metabolism *in vivo*. Until recently, iodination was the only economically feasible approach to studying RBP kinetics. Recombinant protein expression systems may be an ideal way to produce RBP labeled with radioactive or stable isotopes of amino acids and/or retinol labeled with isotopes of hydrogen or carbon. Potentially useful *Escherichia coli* secretion vector systems have recently been described (Sivaprasadarao and Findlay, 1993; Wang *et al.*, 1993). In our own studies (R. Blomhoff, M. H. Green, and colleagues, unpublished results), recombinant labeled RBP produced in an *in vitro* expression system did not act the same in intact rats as *in vivo*-labeled plasma. Similarly we (M. H. Green, J. E. Smith, and colleagues, unpublished results) have observed altered kinetic behavior using *in vitro*-labeled [^{3}H]retinolRBP. Thus isolation/preparation procedures may alter kinetic behavior of RBP or [^{3}H]retinol bound to RBP. For any of these new labeling techniques, the critical things will be to compare kinetics of the labeled moieties to some credible reference (e.g., [^{3}H]retinol-labeled plasma prepared *in vivo*) and then to determine whether any observed differences are due to important biological phenomena or to preparative problems.

IV. WHOLE-BODY MODELS FOR VITAMIN A METABOLISM

As a follow-up to our collaborative study with Underwood's lab (Lewis *et al.*, 1981), we carried out a 35-day *in vivo* turnover study in rats (n = 11) with marginal vitamin A status (liver vitamin A ranged from 100 to

415 nmol) (Green *et al.,* 1985). [^{3}H]Retinol-labeled plasma was administered; short- and long-term tracer and tracee data were collected for plasma (Fig. 2), liver, kidneys, eyes, adrenals, small intestine, lungs, testes, skin, and rest of carcass. For development of an initial model, we lumped all organs other than liver and kidneys with the rest of carcass. Based on prevailing conventional wisdom (Underwood *et al.,* 1979), we postulated a five-compartment starting model. We used the SAAM/CONSAM computer programs and model-based compartmental analysis to compare our data to the initial model. To fit the data, a model with eight physiological compartments was required: one each for plasma retinol and retinyl esters, and two each for liver, kidneys, and rest of carcass (Fig. 3). Many parameters were well identified (i.e., statistical uncertainties were low); others (e.g., those describing liver vitamin A dynamics) were not, indicating areas for future studies (see below).

Several interesting hypotheses resulted from this model (Green *et al.,* 1985). (1) Plasma retinol recycled 12 times before irreversible loss and its turnover rate (nmol/day) was 13 times the disposal rate (24 nmol/day). That is, in support of our previous results (Lewis *et al.,* 1981), an average plasma retinol molecule apparently recycles many times before irreversible utilization. (2) In contrast to the belief that the liver is the sole source of plasma retinol/RBP, our model predicted that 55% of plasma retinol input was from the liver and 45% was from extrahepatic tissues. (3) The model predicted that, in these rats that had marginal liver vitamin A stores and that were in slight negative vitamin A balance, almost half of the whole-

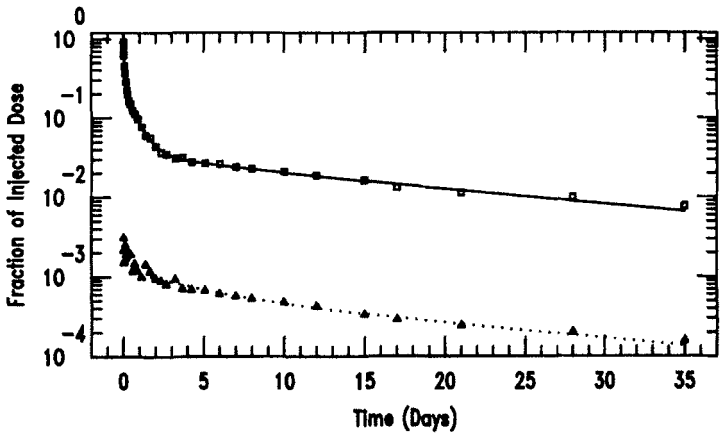

FIG. 2. Observed data and model-simulated values for fraction of dose in plasma retinol (□) and retinyl esters (△) vs time after intravenous administration of [^{3}H]retinol-labeled plasma. The model is shown in Fig. 3; data are from Green *et al.* (1985).

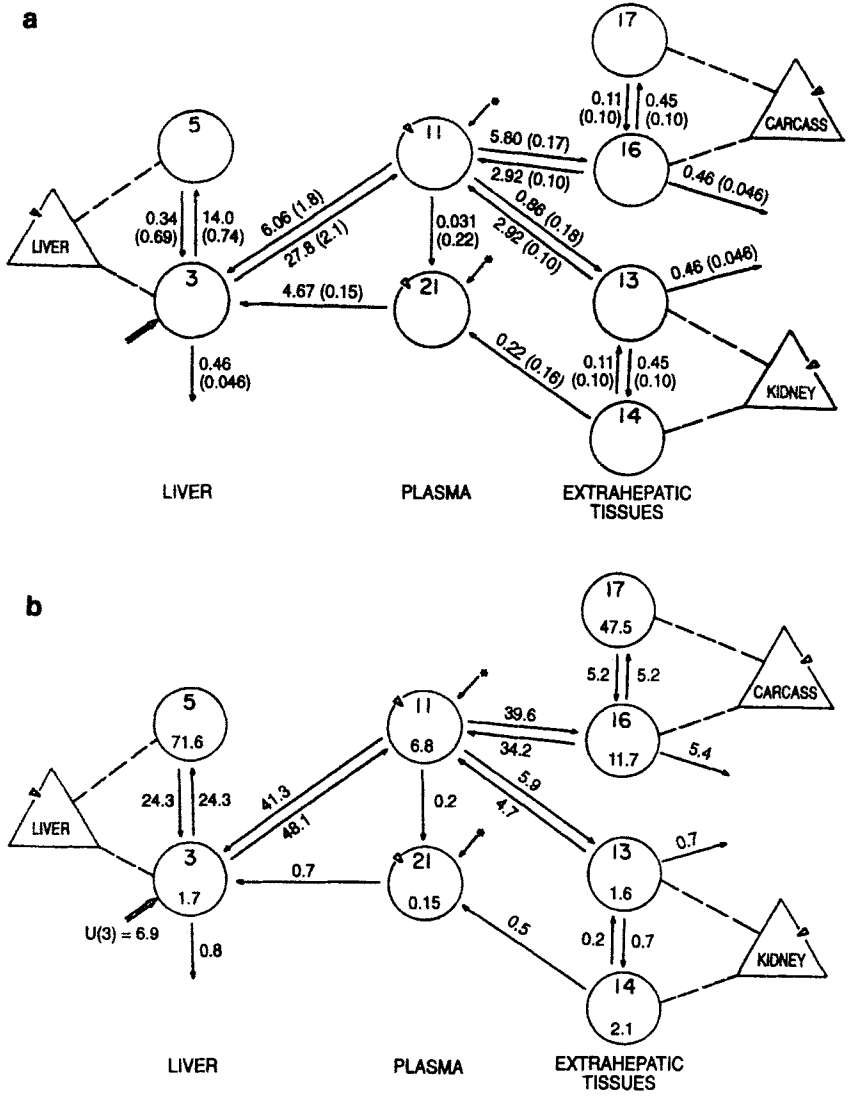

FIG. 3. (a) Model proposed by Green *et al.* (1985) for whole-body vitamin A metabolism in rats. Circles represent compartments, large triangles are functions that sum multiple compartments, small triangles indicate sites of sampling, asterisk shows site of tracer introduction, and wide arrow indicates site of dietary vitamin A input. Compartment 11 is plasma retinol and compartment 21 is plasma retinyl esters. Parameters shown are model-derived fractional transfer coefficients [$L(I,J)$ or the fraction of compartment J's mass transferred to compartment I per day] and estimated fractional standard deviation in parentheses. Irreversible loss, both degradative and/or excretory, was modeled as exiting the faster turning-over compartment in liver, kidneys, and carcass. Since the data were insensitive to changes in magnitude of loss from each site, the three fractional transfer coefficients were set equal [i.e., $L(0,3) = L(0,13) = L(0,16) = 0.46\ d^{-1}$]. (b) Calculated pool sizes (nmol) and mass transfer rates (nmol/day) for the model shown in a.

body vitamin A was in extrahepatic tissues. This finding contradicted the prevailing belief that essentially all of the body's vitamin A is stored in the liver. (4) Although it had been previously speculated that vitamin A may recycle from tissues as retinyl esters in lipoproteins, our analyses showed, and our model predicted, that only a minor amount (0.6%) of the vitamin A recycling through plasma was in the form of retinyl esters. Thus we hypothesized that nearly all of the vitamin A recycles as retinol bound to RBP. This led us and others to wonder what the source of RBP is for retinol recycling: previously it was thought that all of the circulating RBP came from liver parenchymal cells. Since plasma retinol acted kinetically as a single homogeneous compartment, we conclude that both newly secreted retinol/RBP and recycling retinol/RBP behave similarly.

After developing the initial model, we carried out similar but more extensive studies in rats that were vitamin A-deficient (Lewis *et al.*, 1990) or -sufficient (Green and Green, 1987) to investigate how vitamin A status influences vitamin A dynamics. Tracer and tracee data for plasma, liver, kidneys, eyes, testes, lungs, adrenals, small intestine, and rest of carcass were collected for 35 or 115 days (deficient and sufficient rats, respectively) after injection of [^{3}H]retinol-labeled plasma. Tracer data were also collected for urine and feces. Although parts of the data from vitamin A-sufficient rats have been published (Green and Green, 1987; Green *et al.*, 1987, 1992), we continue to work on the development of a complete whole-body model for vitamin A kinetics in these rats.

For the rats with low vitamin A status (liver vitamin A, <8 nmol), plasma retinol turnover rate was 12 times the irreversible disposal rate (5.8 nmol/day) and an average retinol molecule recycled to plasma 12 times before irreversible loss (Lewis *et al.*, 1990). These parameters were similar to the values we had previously reported (Green *et al.*, 1985). The model (Lewis *et al.*, 1990) predicted that only 7.5% of the predicted whole-body vitamin A was in the liver. Forty-four percent of plasma retinol turnover was predicted to be transferred to the kidneys and almost all of this was recycled. The model predicted that 47% of the vitamin A output was via urine and 53% via feces; the irreversible loss data were very useful in identifying other model parameters. The model incorporates metabolite-, urinary-, and fecal-delay elements. More work needs to be done to model the sites of formation and rates of excretion of vitamin A metabolites.

V. EMPIRICAL COMPARTMENTAL ANALYSIS OF VITAMIN A METABOLISM

When we compared the long-term plasma disappearance curves for rats with low, marginal or high vitamin A status (Fig. 4), some interesting

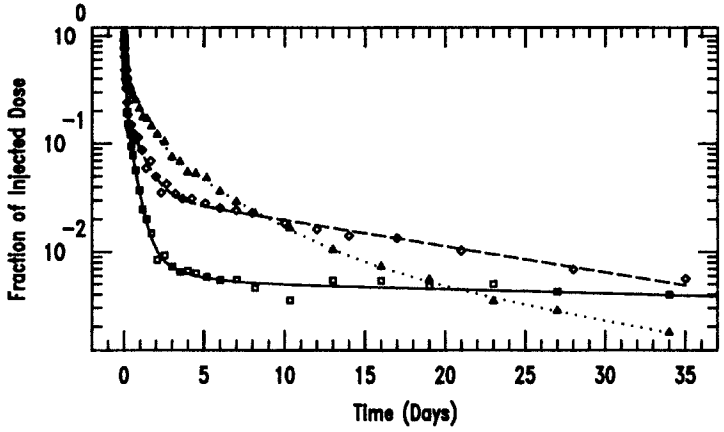

FIG. 4. Observed data and model-simulated values for fraction of dose in plasma vs time after intravenous administration of [^{3}H]retinol-labeled plasma to a rat with high (□), marginal (◇), or low vitamin A status (△). Data are from a randomly selected rat from each group. See Green *et al.* (1987).

differences were apparent. Initial disappearance of tracer from plasma was similar in the three groups, indicating that vitamin A status may not influence plasma retinol transit (turnover) time. Between 3 and 5 days, the disappearance data were very dependent on vitamin A status. That is, the larger the liver vitamin A stores, the lower the plasma fraction of the injected dose left in plasma at that time. This observation was subsequently used to develop predictive equations for estimating liver vitamin A stores using radioactivity in a single plasma sample obtained 36 days after intravenous (Duncan *et al.*, 1993) or oral administration (Adams and Green, 1994) of [^{3}H]retinol. In comparing plasma disappearance data for rats at three levels of vitamin A status, it was also evident that the terminal slope was inversely related to vitamin A status: rats with lower vitamin A reserves dispose of a larger fraction of their vitamin A per day.

To quantitate these effects of vitamin A status on retinol dynamics, we used empirical compartmental analysis (Green *et al.*, 1987). Plasma tracer data vs time were fit to multiexponential equations, and the slopes and intercepts were used to calculate kinetic parameters. In this and subsequent experiments (Green and Green, 1994), a three- or four-component equation was required to fit plasma data. Vitamin A disposal rate, or irreversible utilization, increased with increasing vitamin A intake and status and averaged 4.2, 28, or 41 nmol/day in rats with low, marginal, or high vitamin A status, respectively. The mean sojourn time (the average time a vitamin A molecule spends in the system from the time of entry until irreversible loss)

ranged from 5.5 to 18 to 79 days in the same rats. Since 98% of the variance in mean sojourn time could be predicted by variance in liver vitamin A, we assume that this parameter primarily reflects time spent as retinyl esters in vitamin A stores. Vitamin A status had little effect on plasma retinol turnover time (23 hr), recycling number (79), or plasma retinol fractional catabolic rate (11.3 pools/day). In the rats with low vitamin A status, 32% of the total traced mass of vitamin A was found in the liver. This was similar in rats with marginal vitamin A status, but much lower than in those with high status (107%). That is, in the latter group, liver vitamin A mass determined chemically was greater than the model-predicted total traced mass. Thus it appears that not all of the liver vitamin A was exchangeable with plasma retinol even after 115 days. This observation indicates that some of the vitamin A stored in liver may be physically inaccessible for exchange even after one third of a year.

Recently we used empirical compartmental analysis to develop a general three-compartment model for vitamin A kinetics in rats. A compartment is defined as an amount of material that is well-mixed (i.e., homogeneous) and that turns over more slowly than the time required for mixing within the compartment (Green and Green, 1990a). Plasma tracer response data from three separate studies (Green and Green, 1994; Kelley *et al.*, 1994; Adams *et al.*, 1995) were fit to multiexponential equations to indicate the minimal number of compartments required to model whole-body vitamin A metabolism as viewed from the plasma space. In this three-compartment model (Fig. 5), the central plasma compartment containing retinol bound to RBP/TTR (compartment 1) is the site of input of dietary vitamin A

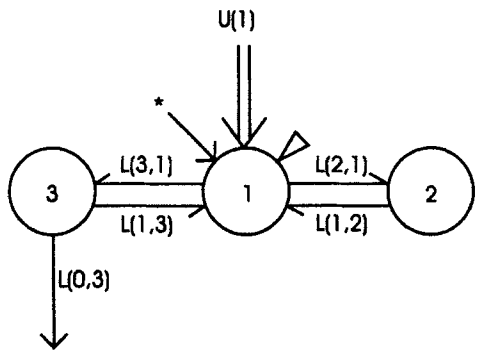

FIG. 5. General 3 compartment model for whole-body vitamin A metabolism. Circles represent compartments, the asterisk is the site of introduction of tracer, $U(1)$ represents dietary vitamin A input, the small triangle indicates the site of sampling. Fractional transfer coefficients $[L(I,J)]$ are defined in the legend to Fig. 3.

after processing of chylomicron vitamin A in the liver and also the site of input of tracer. The transit time for plasma retinol is 12 hr. As in our previous studies, this seems to be relatively independent of vitamin A status. A transit time of 12 hr is sufficient time for tracer to mix with tracee before any significant turnover occurs, thus making this an ideal system for the application of compartmental analysis (Brownell *et al.,* 1968). In this model, plasma retinol exchanges with vitamin A in two extravascular compartments. The larger, slower turning-over pool (compartment 3) is hypothesized to contain slowly turning-over intracellular retinol and retinyl ester stores. The faster turning-over pool (compartment 2) is hypothesized to include vitamin A in interstitial space and that in the process of kidney filtration and reabsorption. As discussed above, and indicated by both empirical and model-based compartmental analysis, there is extensive recycling of plasma retinol. About two-thirds of the plasma retinol output is to compartment 2 and the rest to compartment 3. Based on estimates of fractional transfer coefficients from our modeling, we predict that it is RBP-bound retinol that leaves the plasma, rather than free retinol or retinol/RBP/TTR (Blomhoff *et al.,* 1991). To fit available data, system output had to occur from the large, slow turning-over extravascular pool and input of newly absorbed vitamin A had to be into the plasma retinol pool. This indicates that most of the newly absorbed vitamin A is processed through plasma RBP before irreversible loss. Transit time for retinol in the small, faster turning-over extravascular compartment is 34 hr, compared to 910 days in compartment 3 for rats with moderate vitamin A status and 70 days for those with high status. Although this "working hypothesis" three-compartment model adequately fits plasma retinol turnover data from three different experiments, its structure and interconnectivities, and the physiological correspondence of the pools that compose the two extravascular vitamin A compartments, require validation in future studies.

VI. LIVER VITAMIN A METABOLISM

A. COMPARTMENTAL MODEL

The liver has long been known to be an important organ in whole-body vitamin A metabolism, and the role of various hepatic cell types in vitamin A metabolism was a topic of controversy for many years. When our initial modeling studies on whole-body vitamin A metabolism suggested that this approach might be ideal for characterizing liver vitamin A dynamics, we were fortunate to begin a long-term collaboration with colleagues (especially R. Blomhoff, K. R. Norum, and T. Berg) at the University of Oslo

in Oslo, Norway. Their expertises in liver cell fractionation and vitamin A metabolism have provided unique data for modeling. In 1985/86, we carried out two studies in Oslo to model the dynamics of vitamin A metabolism in rat liver: in one, [^{3}H]retinol-labeled plasma was administered as in our earlier studies; in the second, [^{3}H]vitamin A-labeled chylomicrons were used to trace newly absorbed dietary vitamin A. In both studies, we separated hepatic parenchymal cells (PC) from whole liver and analyzed liver and liver PC for total retinol and unesterified retinol mass and radioactivity. Plasma and rest of carcass radioactivity were measured for 97 days after administration of [^{3}H]retinol-labeled plasma and 49 days after injection of [^{3}H]vitamin A-labeled chylomicrons. Data for the first 15 days from both experiments were analyzed by model-based compartmental analysis and a single, 11-compartment "working hypothesis" model was developed to describe whole-body vitamin A dynamics subsequent to clearance of chylomicron vitamin A by the liver (Fig. 6) (Green *et al.*, 1993). Data on vitamin A masses were well predicted by the model, and these data were very useful in constraining the model during model development. As in our previous models, plasma retinol data fit one compartment, and two compartments were required in carcass. Eight compartments were required to describe liver vitamin A dynamics. Although liver vitamin A levels differed between the rats that received [^{3}H]retinol-labeled plasma and

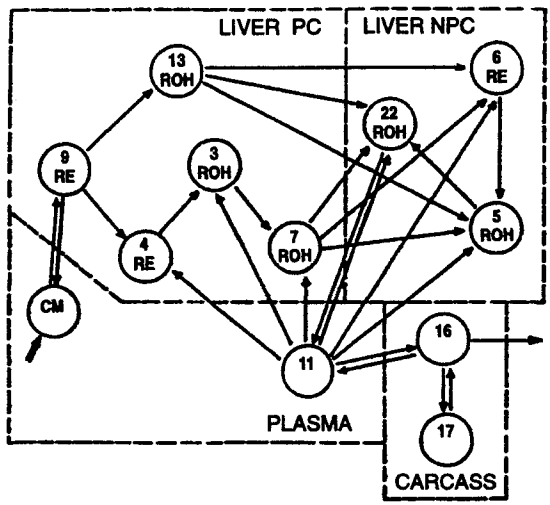

FIG. 6. Compartmental model proposed by Green *et al.* (1993) for liver and whole-body vitamin A metabolism. Compartment 11 is plasma retinol. PC, parenchymal cells; NPC, nonparenchymal cells (assumed here to be perisinusoidal stellate cells); ROH, retinol; RE, retinyl esters; CM, chylomicrons.

[^{3}H]vitamin A-labeled chylomicrons, values for only 3 of the 24 fractional transfer coefficients had to differ to fit the two data sets. Thus we speculate that these three parameters [L(5,6), L(3,4), and L(22,5); Fig. 6] are sensitive to vitamin A status. This makes physiological sense since these parameters reflect retinyl ester hydrolysis and stellate cell retinol secretion.

This was a challenging project, partly because we needed to develop a technique for accurately estimating whole liver and total PC tracer and tracee in the same rat. Equally difficult was the fact that the model developed to fit the data predicted several contradictions to conventional wisdom. In keeping with prevailing assumptions, our initial model indicated that PC are the major site of hepatic RBP production and RBP/retinol secretion. To fit the data, an interaction with nonparenchymal cells (presumably perisinusoidal stellate cells, the site of liver vitamin A storage) was required. We hypothesized that there is an efficient transfer of retinol (presumably bound to RBP; Blomhoff *et al.*, 1988) from PC to stellate cells (SC). Based on our data and the model developed, we hypothesize that about 50% of the plasma retinol that recycles to the liver in rats at these levels of vitamin A status is taken up by PC and the rest by SC. This distribution of retinol recycling to liver is interesting, since PC comprise 6065% of liver cell number whereas SC account for only 7%; SC are smaller in size than PC. Thus the number of putative RBP receptors on stellate cells may be much higher than on PC. The idea that liver perisinusoidal stellate cells may secrete retinol/RBP into plasma without a prior transfer to liver PC is still not fully accepted, but colleagues in the field agree that the modeling approach has generated a good hypothesis for future research.

Fifteen to 30 min after administration of [^{3}H]vitamin A-labeled chylomicrons, there was a decrease in the slope of the plasma response curve for [^{3}H]retinyl esters (Fig. 7) which we interpreted as indicating recycling of chylomicron remnants, possibly those that had been trapped in liver sinusoids. Modeling the data from a previous chylomicron turnover study (J. E. Balmer, J. B. Green, and M. H. Green, unpublished results) indicated that about one third of [^{3}H]cholesterol-labeled chylomicron remnants temporarily recycle back to plasma while the remainder are cleared by liver PC. In view of the size of chylomicrons, it is hard to imagine that they are endocytosed by a mechanism similar to receptor-mediated endocytosis for low density lipoproteins. Our model (Fig. 6) suggests that, after chylomicron retinyl esters are hydrolyzed soon after interaction with liver PC, some of the retinol is rapidly reesterified or transferred to stellate cells. In SC it can quickly move into a retinyl ester pool, presumably by action of lecithin:retinol acyltransferase, or enter a slow turning-over retinol pool, presumably bound to cellular retinol-binding protein (CRBP), or be secreted into plasma bound to RBP. This trifurcation of retinol into three

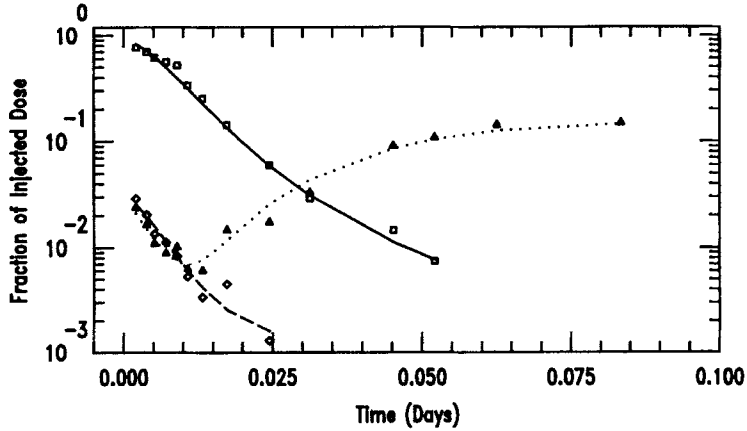

FIG. 7. Observed data and model-simulated values for fraction of dose in plasma long-chain retinyl esters (□), retinol (△), and retinyl acetate (◇) vs time after intravenous administration of [^{3}H]vitamin A-labeled chylomicrons. Data are from Green *et al.* (1993).

kinetically distinct pools following transfer from PC to SC occurs very rapidly (i.e., in less than 10 min) and presumably occurs at the plasma membrane of stellate cells. A similar trifurcation is predicted to occur for retinol recycling from the plasma RBP pool back to liver PC or SC (Fig. 6).

To mobilize liver stores, our model predicts that retinyl esters in PC or SC are hydrolyzed and the resulting retinol is transferred to the slower turning-over retinol pool (compartment 5 in SC and 3 in PC), presumably bound to CRBP. It is then transferred to an exocytosis compartment. Our kinetic data indicate that this retinol does not need to go back to PC before secretion into plasma. Maybe apoRBP can interact with RBP receptors and equilibrate retinol between intracellular CRBP and plasma RBP. If so, this is an excellent example of homeostatic control since cellular retinol pools are in equilibrium with plasma retinol. If an exchange of retinol between apoCRBP and apoRBP is shown to be mediated by a specific membrane protein, the protein should perhaps be named a retinol transporter, rather than an RBP receptor (Blomhoff *et al.*, 1991).

B. STORES

Textbooks indicate that 8590% of the body's vitamin A is stored in the liver. Although there is no doubt that the amount of vitamin A in liver is higher than in other organs in the vitamin A-sufficient state, our modeling studies predict that extrahepatic stores of vitamin A can be significant. In our several kinetic studies, we calculated the total traced mass of vitamin

A or the amount of vitamin A that equilibrates with [^{3}H]retinol introduced into plasma. When using model-based compartmental analysis, total traced mass is obtained as the sum of the individual compartment masses; using empirical compartmental analysis, total traced mass is calculated as the product of mean sojourn time and disposal rate. When we recently compared the calculated total traced mass to the measured liver vitamin A level for 62 rats that had a wide range of liver vitamin A levels and had been studied in various kinetic experiments (Adams *et al.*, 1995; Green *et al.*, 1985, 1987; Green and Green, 1987, 1994; Kelley *et al.*, 1994; Lewis *et al.*, 1990), it was apparent that, even at very low liver levels (1500 nmol), there are appreciable extrahepatic reserves of vitamin A (Fig. 8). This extrahepatic pool seems to get depleted even more slowly than the liver vitamin A pool in response to a lowered dietary input. As liver vitamin A increases, so do the extrahepatic stores. This replenishment of extrahepatic stores when there is adequate vitamin A may be similar to the muscle glycogen system. At very high liver levels (>3000 nmol), the model-predicted total traced mass underestimates measured liver vitamin A levels, as if some of the vitamin is in a nonexchangeable pool in stellate cells. Even when turnover studies were carried out for 115 days in vitamin A-sufficient rats, tracer did not fully equilibrate with this pool.

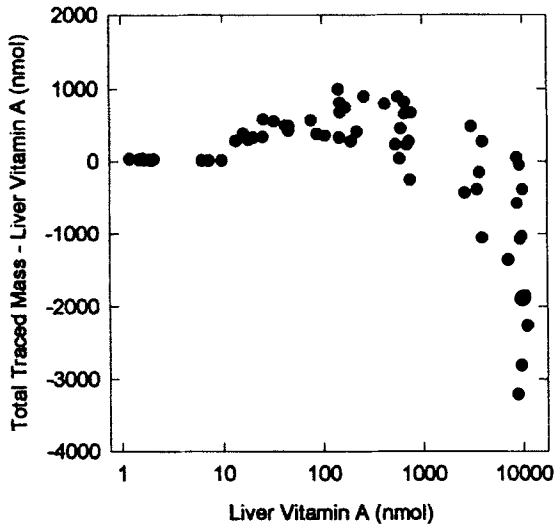

FIG. 8. Observed values for liver vitamin A mass vs model-predicted total traced mass minus observed liver vitamin A mass.

VII. OTHER TISSUES

In order to develop a whole-body model for vitamin A metabolism which incorporates dynamics in many tissues, a large number of data points from plasma, urine, feces, and individual organs must be modeled simultaneously. Needless to say, this juggling has proven to be a challenging exercise. An alternate modeling approachthe "forcing function"has proven useful and more straightforward for describing vitamin A dynamics in individual organs (Green *et al.*, 1992). The forcing function approach has been used by other investigators to model zinc metabolism in humans (Foster *et al.*, 1979) and is applicable to many different systems. This technique makes use of the fact that, since plasma is the transport medium for vitamin A (and likewise many other nutrients), it provides the vitamin A input to individual tissues. This fact allows us to "uncouple" individual organs from the whole system and makes model development more manageable. To use the forcing function approach, long-term data for plasma [^{3}H]retinol turnover are fit to a multiexponential equation to define the temporal plasma tracer response. This function is then used to "drive" tracer input into individual tissues. The output from the tissue or organ being modeled is to unknown site(s) outside the organ. Although one cannot determine how much of the vitamin is recycled to plasma vs irreversibly metabolized, useful information is obtained on fractional transfer coefficients describing uptake of vitamin A by the tissue, within the tissue and out of the tissue.

When we applied the forcing function approach to our data on vitamin A dynamics in organs of rats at three levels of vitamin A status, several interesting results were obtained (Green *et al.*, 1992). For the eyes, a one compartment model was adequate to fit data at all three levels of vitamin A status (Fig. 9). The model indicated that only ~0.1% of plasma vitamin A turnover goes to the eyes. If all of this vitamin A is used irreversibly for visual, neuronal, epithelial tissue and other functions of vitamin A, it represents only ~1% of whole-body disposal rate, even in vitamin A-deficient rats. In such rats, the 0.06% of plasma retinol turnover that went to the eyes represented almost a 90% reduction in the rate of retinol transfer to the eyes. However, the eyes conserved ~50% of normal eye vitamin A content and the transit time (75 days) was six times longer than in the other two groups. Thus, it appears that, in vitamin A-depleted rats, the eye can down-regulate its uptake and turnover of vitamin A. When the forcing function approach was used to describe vitamin A metabolism in the small intestine, a two-compartment model was required. The model predicted that vitamin A transfer to this organ is appreciable: 14 nmol/day in normal rats or 3.4% of plasma retinol turnover. This high rate of transfer, which

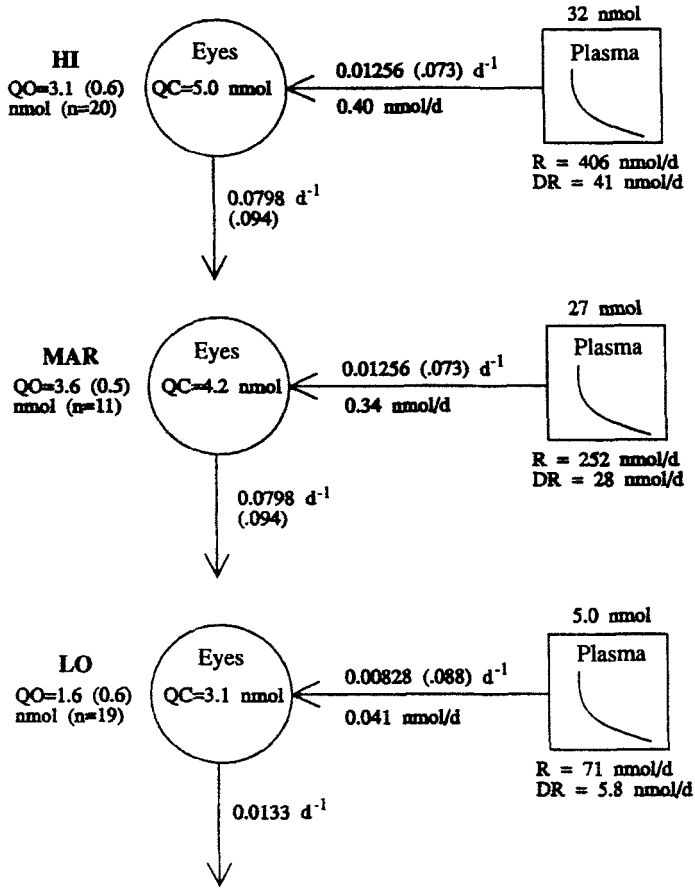

FIG. 9. One-compartment model for vitamin A metabolism in eyes of rats with high (HI), marginal (MAR), or low vitamin A status (LO). On the right are the plasma [^{3}H]retinol forcing function (box), the plasma retinol pool size (above box), plasma retinol transfer rate (R), and the whole-body vitamin A disposal rate (DR). Data shown with interconnectivities are model-predicted fractional transfer coefficients and their estimated fractional standard deviation (FSD) and retinol transfer rates (nmol/day). No FSD is given for the low group because turnover of vitamin A from eyes of rats in this group was so slow that the output rate constant could not be predicted with confidence. Also shown are observed (QO) and model-predicted total retinol masses (QC). Reprinted from Green *et al.* (1992) by courtesy of Marcel Dekker, Inc.

is surpassed only by that to kidneys and liver, may reflect the importance of vitamin A in maintenance of epithelial tissue.

Modeling has also revealed some interesting ideas about the contribution of the kidneys to whole-body vitamin A metabolism. In our first model

(Green *et al.*, 1985), kidneys were sampled 1, 2, 15, and 35 days after administration of [^{3}H]retinol-labeled plasma. The model developed to fit the data predicted that only 7% of plasma retinol turnover went to the kidneys and that the average retinol molecule did not pass through kidney tubules. Since this finding was unexpected in view of work by other investigators, we simulated kidney filtration and reabsorption using CONSAM and speculated that earlier samples were needed to adequately model the role of the kidneys in vitamin A metabolism. Thus in the study of rats with low vitamin A status (Lewis *et al.*, 1990), kidneys and other organs were first sampled 12 min after dose administration. The model predicted that 44% of the plasma retinol turnover was transferred to the kidneys. If all of this were lost, it would be seven times the whole-body disposal rate. Thus essentially all of the filtered retinol must be reabsorbed and recycled to plasma. Analyses done with our first modeling study (Green *et al.*, 1985) indicated that plasma lipoproteins were not the vehicle for retinol recycling. Work in other labs (Soprano *et al.*, 1986) has shown that the S3 segment of the kidney contains messenger RNA for RBP, but that is not the correct anatomic site for reabsorption. Perhaps the proximal tubule reabsorbs retinol bound to RBP instead of degrading the filtered RBP.

VIII. VITAMIN A DISPOSAL RATE

A final, more philosophical issue has been raised by our modeling studies and is one of the topics we are currently pursuing. As we have quantified vitamin A disposal rate at various levels of vitamin A status, we have been led to wonder why the body disposes of so much of a valuable essential nutrient such as vitamin A. Perhaps this is an evolutionary response to the fact that we are an open system. That is, since we consume essential nutrients such as vitamin A in the diet, we need to have physiological or biochemical means to eliminate them.

To look at the determinants of vitamin A disposal rate, we used multiple regression analysis to relate our data on disposal rate for 62 rats to vitamin A intake, liver vitamin A levels, and plasma retinol pool size (Fig. 10) (Kelley *et al.*, 1994). These three variables predict 91% of the variation in disposal rate; 68% of the reduction in sum of squares comes from plasma retinol, 18% from liver vitamin A, and 14% from vitamin A intake. Our data indicate that disposal rate does not fall until liver vitamin A levels are essentially depleted. That is, as long as plasma retinol levels are normal, degradation rate is high, implying a nonfunctional utilization of the vitamin. We call this "degradative preservation." We conclude that plasma retinol is the major determinant of vitamin A disposal rate and hypothesize that

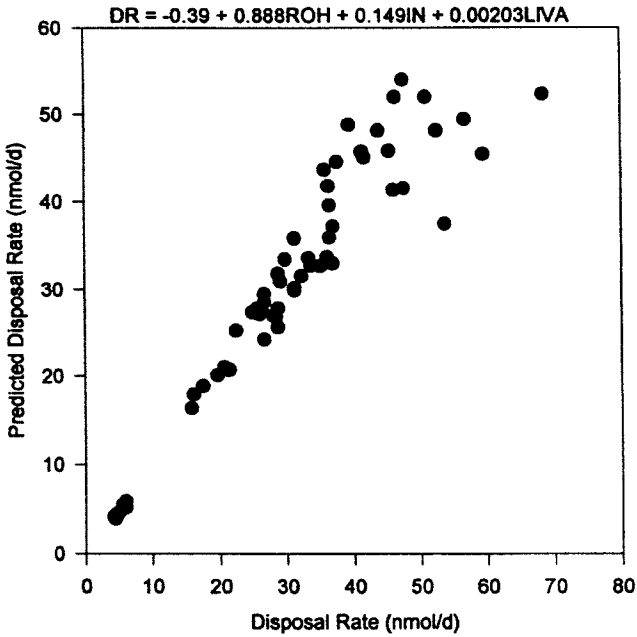

FIG. 10. Relationship between vitamin A disposal rate (DR; nmol/day), plasma retinol pool size (ROH; nmol), vitamin A intake (IN; nmol/day), and liver vitamin A (LIVA; nmol).

degradation may be driven by an intracellular pool of vitamin A in equilibrium with plasma retinol. Assuming availability of research funds, we will continue to use compartmental analysis to study vitamin A metabolism and to validate the hypotheses presented here.

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