VITAMIN A METABOLISM, KINETIC BEHAVIOR AND UTILIZATION: RATIONALE FOR THE CONTINUED DEVELOPMENT AND USE OF AN ISOTOPE DILUTION TECHNIQUE FOR ASSESSING VITAMIN A STORES IN HUMAN POPULATIONS

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1. INTRODUCTION AND SCIENTIFIC BACKGROUND

My purpose here is to discuss the applicability of isotope dilution methods to the assessment of vitamin A status in humans. First, I will briefly review the oral isotope dilution method for assessing vitamin A stores [1,2]. Then I will highlight some aspects of vitamin A intake and metabolism that relate to the oral isotope dilution method. Next I would like to propose some areas of methodological research and development that I feel are needed to advance, validate and improve this technique. Finally, I will make some comments about issues that may require our special attention as we plan stable isotope studies during pregnancy and lactation.

Before reviewing the oral isotope dilution method, I would like to remind us of the concept on which isotope dilution methods are based and to rethink the use of the term "isotope equilibrium." Regarding the original concept, we need to remember that it was developed for a closed system. If, for example, one added a known amount of dye to a beaker of water, then thoroughly mixed the solution and determined the concentration of the dye, one could calculate the volume of water present in the beaker as

volume of water = dye added / equilibrium dye
(mL) (mg) concentration (mg/mL).

Obviously, in real life we will not be studying a closed system that has reached an isotopic steady state: humans are open systems in a non-equilibrium- and typically also a non-steady state. Fortunately, as I will discuss later, meaningful advances have been made in adapting the isotope dilution method to dynamic open systems, but we must always be aware of the assumptions underlying the original equation.

Also, I propose that we stop using the term "equilibrium" (as in "isotope equilibrium") when we are using this type of methodology for *in vivo* experiments. By definition, an isotope is said to be in equilibrium when the rate of input of tracer equals its rate of output, and the specific activity ratio of tracer/tracee has reached a plateau in the system. Because of the dynamic behavior of vitamin A, such a state does not typically exist with respect to administered tracers for vitamin A, except in special situations resulting from a long period of continual administration of the tracer. In a more usual case, tracer disappearance will follow parallel kinetics (but at different specific activities) in all possible sampling sites, once the vitamin has thoroughly mixed with the exchangeable pools of vitamin A. This fact is often referred to in the tracer kinetics literature as the precursor-product relationship [3].

Now I would like to discuss what we are calling the oral isotope dilution method. I will present the equation published by Furr *et al.* [2] and describe how the authors intended it to be used to estimate total liver vitamin A in projects such as those we are planning at this workshop. The equation is: TLR = F * dose * (s * a * [H/D - 1]).

TLR is the total liver reserve of vitamin A (nmol). F is the fraction of an orally administered dose of deuterated vitamin A that is absorbed and retained in the liver; blood must be sampled long enough after dosing to allow the label to have mixed with

endogenous stores. In previous studies (1,4), blood was collected at 14 or 26 d, respectively. F is most commonly assumed to be 0.5, and has been shown to range in rats from ~0.13 to >0.8 at low versus high liver vitamin A levels, and to average 0.49 ± 0.05 (mean ± SD) in sheep [1]. Dose is the mass of deuterated retinol in the administered dose (mmol). The parameter **s** is the ratio of the specific activities (D/H) of retinol in serum to liver at the time of blood sampling. Often people incorrectly equate the D/H stable isotope ratio with tracer specific activity (dpm/mass) As pointed out by Cobelli *et al.* [4], a transformation of the data is required to arrive at an equivalent entity. The value for **s** was determined to be 0.65 ± 0.15 in rats [5] and 0.68 ± 0.27 (mean ± SD) in humans [2]. **a** is a factor that was added by Furr *et al.* [2] to account for the disappearance of tracer from the body during the mixing period and was calculated as

$$a = e^{-kt}$$

where **k** = 1/140 d (140 days is the estimated half-life of vitamin A turnover in the liver) and **t** is the time (d) at which the blood sample is taken following dose administration. Typically, half-life ($t_{1/2}$) is converted into a rate constant by using the following equations:

$$y(t) = y(t_0)e^{-kt} \text{ and}$$

$$0.5 = 1 * e^{-kt(1/2)}.$$

Solving for **k**, one obtains $k = \ln 2/t_{1/2}$. Sauberlich *et al.* [6] measured the $t_{1/2}$ for vitamin A to average 144 days (range, 75 to 241) in 8 adult male volunteers. Therefore, **k** would equal 0.0048 day^{-1} (range, 0.0029 to 0.0092). H/D is the ratio of ^1H to ^2H in plasma retinol at the time of sampling. Finally by subtracting 1 in the equation for TLR, one removes the contribution of the deuterated retinol dose from TLR. That is, the equation calculates the pre-dose total liver reserve.

If TLR is being calculated at two times (e.g., before and after a vitamin A supplementation regimen and one discovers in the first test that the dose caused a substantial perturbation of TLR, then **a** must be computed from the first to the second determination of TLR. The equation for comparing the first TLR with the second becomes

$$TLR = F * dose * (s * a * [H_i D + 1]).$$

Based on the above discussion, it is clear that the coefficients (βs) in the regression equation of Furr *et al.* (i.e., F, s and a) are not constants but rather unknowns with certain confidence intervals. As I will mention again later, this leads us into an area of multiple regression called by some "constrained multiple regression". As the equation is currently being applied (i.e., using the mean values for these regression coefficients), it will provide more accurate prediction of liver stores for groups of individuals that it will for individuals in a particular group.

2. VITAMIN A METABOLISM AND THE ISOTOPE DILUTION METHOD

Vitamin A metabolism and kinetic behavior have been discussed in detail in several recent reviews [7,8]. Here I will briefly overview these topics and emphasize particular features that will affect results of the studies we are planning. First, we need to consider the effects on vitamin A dynamics of the amount of vitamin that will be administered in order to label the tracee system with enough isotope for accurate, long-term detection. Because of the currently-known analytical detection limits for the

available stable isotopes of vitamin A, investigators will be forced to administer a dose (>3 mg) that will perturb the underlying tracee system (that is, the dose will be large enough to alter kinetics of endogenous vitamin A and body vitamin A stores). To a kineticist, this represents a solvable but definitely more complex situation than administration of tracer amounts of a compound, as could be done with tritiated vitamin A.

One "extra-metabolic" feature that requires serious thought is dietary intake of vitamin A during the field study. We will consider the ramifications of level of intake below, but I note here that it will be very useful to have accurate estimates of intake during the study, since we know that the newest dietary vitamin A entering the liver is the first out into the blood [9,10]. This occurrence will dilute the plasma D:H ratio.

Several characteristics of vitamin A metabolism *per se* may have an impact on use of the oral isotope dilution method. One consideration is that vitamin A absorption is variable (e.g., definitely among individuals, and possibly as a function of fat intake, vitamin A status and level of vitamin A intake). In studies using lymph-duct cannulated rats [11], we determined that the absorption efficiency for vitamin A averaged 76% ± 16 (mean ± SD, n=11). That is, the coefficient of variation was high (21%). More work needs to be done to develop methods for determining vitamin A absorption efficiency in a given individual, as well as the efficiency of bioconversion of carotenoids to vitamin A.

Fig. 1 presents a simplified overview of vitamin A metabolism; see references 7 and 8 for more details. Briefly, preformed dietary vitamin A or provitamin A carotenoids are absorbed into the enterocytes of the upper small intestine as retinol and carotenoids, respectively. There, a portion of the carotenoids will be converted to retinol. Retinol is esterified, primarily by the enzyme lecithin:retinol acyltransferase at the endoplasmic reticulum, which is the site of production of absorptive lipoproteins (chylomicrons). The retinyl esters will partition into the core of the chylomicron along with cholesteryl esters and triacylglycerols. Nascent chylomicrons are processed through the enterocyte and secreted from the cell by exocytosis. Since chylomicrons are too large to fit through interendothelial spaces or fenestrations in the capillary wall, they enter the lymphatic system and are transported through the thoracic duct to plasma via the subclavian vein in the shoulder. In plasma, chylomicrons acquire apolipoprotein CII from high density lipoproteins and will begin to be metabolized by the enzyme lipoprotein lipase (LpL) which is located on the endothelial surface of capillaries (primarily in adipose tissue, muscle and lactating mammary glands). LpL action accounts for the extrahepatic removal of ~90% of chylomicron triacylglycerols and variable amounts of vitamin A. This uptake of vitamin A may be via monomolecular transfer or particle uptake by extrahepatic tissues. Such an extrahepatic clearance of chylomicron vitamin A may be an important route of delivery of vitamin A to mammary tissue, especially during late pregnancy and lactation. This possibility needs to be considered when using isotope dilution methods in pregnant/lactating women.

The remaining "dilapidated" chylomicrons (chylomicron remnants) are then cleared by the liver parenchymal cells (hepatocytes) via receptor-mediated endocytosis. The retinyl esters are hydrolyzed and the resulting retinol is either bound to apoRBP (retinol-binding protein) in the endoplasmic reticulum for secretion into plasma or transferred to liver perisinusoidal stellate cells for storage as retinyl esters. The fate of retinol in the hepatocytes depends on the load of vitamin A entering the liver: if the dose is large, as will be the case in our studies as currently proposed, some of the newly-arrived retinol will be re-esterified in hepatocytes, waiting its turn for an opportunity to be transferred out on RBP. This delay in export from hepatocytes may be hours to days, depending on the incoming load of vitamin A. Based on studies in

animals, it is likely that, in subjects with more adequate liver stores, more of the dose will be sequestered in stellate cell retinyl ester pools, whereas in the vitamin A-deficient state, most of the dose taken up into hepatocytes as part of chylomicron remnants will be exported directly into plasma. Vitamin A metabolism by the two cell types will be affected by level of dietary intake during the isotope dilution test and will be perturbed by the orally-administered dose of labeled vitamin A.

In stellate cells, stored retinyl esters become a source of plasma retinol [10]. Stellate cell retinyl esters undergo a slow but continuous hydrolysis and re-esterification before secretion. It may be the plasma apoRBP to holoRBP (holoRBP is retinol bound to RBP) ratio, and the stellate cell apoCRBP (cellular retinol-binding protein) to holoCRBP ratio, that help determine the timing of mobilization of retinol from stellate cells. An important fact for our proposed studies is that there is a continual turnover of stellate cell retinyl ester stores that results in the mixing of "new" vitamin A with "old." As discussed below, it has also been noted that as stellate cell vitamin A stores get large (i.e., at higher and higher levels of vitamin A status), mixing of "new" and "old" is incomplete. Thus for our purposes, we need to keep in mind that some of the retinyl esters may not mix and exchange in a reasonable amount of time (e.g., <50 days) with the administered stable isotope ("new" vitamin A). This possibility will affect the calculation of isotope dilution, as was observed by Bausch and Rietz in rats [1].

Plasma transport, recycling, tissue uptake and utilization of vitamin A are also dependent on vitamin A status. Let's begin with data from animal studies [12,13], in which we know not only vitamin A intake but also the liver vitamin A stores, in order to get some insights into the influence of vitamin A status on the kinetic behavior of vitamin A. Rats at one of four levels of vitamin A nutriture (liver levels of ~8 to ~9700 nmol) received a nonperturbing IV dose of [^{3}H]retinol-labeled plasma that had been prepared in donor rats. The disappearance of administered [^{3}H]retinol from plasma is shown in Fig. 2. The terminal slope of the curves is proportional to the fraction of the exchangeable pool of vitamin A that is irreversibly utilized per day and is inversely proportional to the liver stores of vitamin A. The figure inset reveals that, initially (before 12 h after dose administration), there is little influence of vitamin A stores on the kinetic behavior of the vitamin. By ~3-4 d after dosing, the maximum divergence, reflecting differences in liver vitamin A stores, is evident. Soon (by 7-10 d), this divergence is lost at lower liver reserves of vitamin A. Let me also point out that we have used early data (3-6 d post dosing) from this type of study to develop prediction equations for estimating liver vitamin A levels based on a single blood sample after both IV [14] and oral administration of [^{3}H]retinol [15].

Another impact of vitamin A status on vitamin A kinetics is illustrated in Fig. 3 which provides information on the recycling of [^{3}H]retinol from extravascular pools in rats with high or low liver reserves of vitamin A. Plasma tracer disappearance data from rats with low or high liver vitamin A levels were fit to a 3-compartment model (Fig. 3) which is postulated to represent the central plasma retinol compartment exchanging with two extravascular vitamin A pools (one slowly turning-over and the other fast turning-over). When liver stores are low, the model predicts that there is extensive recycling of the label from the fast turning-over extravascular vitamin A pool and very little dilution of the label in the small, slow turning-over extravascular (storage) pool. Thus the labeled material quickly mixes with body pools of vitamin A and it leaves the system relatively rapidly via degradative pathways. In contrast, in rats with large stores of vitamin A, the portion (~1/3 of the plasma retinol turnover) of the tracer that goes to the slow turning-over pool gets sequestered there and thus retinol entering the plasma from this pool is of low specific activity. As a consequence, the input of tracer into plasma from both extravascular pools is reduced, in comparison to rats with low vitamin A status. For our purposes, it is important to note that, if such an extensive recycling of retinol to plasma

(~8 to 13 times before irreversible utilization) did not occur, the effects of vitamin A stores on plasma retinol specific activity might not be seen.

Similar kinetic behavior has been observed in the limited data that are available for vitamin A turnover in humans. Older data collected by the late DeWitt Goodman are shown in Fig. 4. Three adult subjects received an IV dose of [^{14}C]retinol; disappearance of label from plasma was monitored for up to 240 d. Unfortunately, no blood samples were collected during the first 18 h after dose administration. Note that the pattern of disappearance is similar to that which we had observed in rats with adequate stores (Fig. 2). The average terminal slope of the tracer disappearance curves was 0.58%/day, indicating that <1% of the body exchangeable pool of vitamin A was irreversibly utilized each day. We [9] used model-based compartmental analysis with the conversational version (CONSAM [16] of the Simulation, Analysis and Modeling computer program (SAAM) [17] to fit these data to a three compartment model (Fig. 5). By simulating the response predicted by this model, we estimate that the fraction of the ^{14}C dose administered peaked in the fast turning-over extravascular pool (compartment 3 in Fig. 5) at 0.6 d; that for the first 5 d, almost all of the input of tracer to plasma was from this pool; that tracer peaked in the slow turning-over extravascular pool (compartment 2, the storage pool) at 10 d (55% of the dose) but most of what was going to get there had arrived by 2-3 d (47% of the dose). These predictions emphasize again the extensive recycling of retinol to plasma from the liver and extrahepatic tissues prior to irreversible loss, as well as the mixing of plasma retinol with liver stores of retinyl esters.

In addition to the data of Goodman, I have more recently had the opportunity to work with the as-yet unpublished work of Doris von Reinersdorff (Hoffmann-La Roche, Nutley, NJ). Since these data are not yet published, I will show an example here at the workshop but will not include data figures in the report. $^{13}C_3$-Labeled vitamin A (105,000 nmol [30 mg]) was administered orally to 11 subjects; the appearance and disappearance of retinyl esters, retinol (^{12}C and ^{13}C), and retinol metabolites were followed in plasma for 7 d. Currently we are developing a multicompartmental model to interpret these unique and valuable data. Based on results to date, the model predicts that 55% of the dose was absorbed in this example subject. Both the data and the model demonstrate how such a large load of labeled vitamin A perturbs the underlying tracee system. A less dramatic perturbation will occur using doses we plan to administer in the field studies being discussed at this workshop.

In the experiments we are planning at this meeting, we hope to make use of the observation [15] that there is a "window of separation" in the fraction of a dose of vitamin A in plasma between 3 and 6 days after administration of an oral dose of labeled vitamin A to rats (Fig. 6). This separation is due to differences in the liver reserves of vitamin A (Fig. 7). Non-linear regression was applied to the data shown in Fig. 7 in order to develop a prediction equation for liver vitamin A content:

$$\text{LIV A (nmol)} = 58{,}577\ \text{EXP}(-2715 * FD_{P3D}) + 1810\ \text{EXP}(-127 * FD_{P3D})$$

where EXP is the base **e** of the natural logarithm, and FD_{P3D} is the fraction of the oral dose of [^{3}H]retinol in plasma at 3 d. The Spearman rank correlation (r_s) between observed and predicted liver vitamin A reserves over this large range (5 to 22,000 nmol or 0.48 to 1629 nmol/g) was 0.982. Thus, at 3 d after dosing, at least in rats, there is a sensitive inverse relationship between liver vitamin A levels and the fraction of an oral dose of labeled vitamin A present in plasma. Although the relationship is not linear, the correlation coefficient (r) is 0.509 ($P<0.01$). I will return to this point later when discussing various possibilities for assessing liver vitamin A stores after an oral dose.

Over the years, one of the main goals of my laboratory's research on the kinetic behavior of vitamin A has been to elucidate the determinants of vitamin A requirements. From an input-output perspective, these determinants relate to food preparation, availability of vitamin A from the diet, absorption efficiency and utilization rate. One of the kinetic parameters we calculate from data collected in *in vivo* turnover studies is the disposal rate of vitamin A (i.e., the rate at which vitamin A is irreversibly utilized to fulfill vitamin A function and non-vitamin A-related degradative processes). In Fig. 8, I present the relationship between plasma retinol levels and vitamin A disposal rate [13]. These data combine the results from several published and unpublished studies done in my lab with rats at different levels of vitamin A nutriture. Note in Fig. 8 that, as plasma retinol increases, there is an increase in vitamin A utilization. At the present time, we believe that only a small fraction of this can be attributed to vitamin A-related functional utilization, while the majority may be what we call "degradative preservation". This possibility is particularly important for open systems: if a substance is taken in, there had better be a means of disposing of it. Thus, although mammals are quite efficient at storing vitamin A, at least one species among this class (i.e., rats) is able to vary vitamin A degradation rates. In this regard, I appreciate the comment made by Moore [18] in his classic 1957 text: "In interpreting observations on the rate of fall in the reserves of vitamin we may then be able to decide how much of the vitamin was being used for physiological purposes, and how much is 'running to waste'".

Concluding the discussion of determinants of vitamin A utilization, in Fig. 9 is shown data and a multiple regression equation we have developed [13] to predict vitamin A disposal rate in rats ($n=62$) as a function of plasma retinol, vitamin A intake and liver vitamin A levels. The coefficient of multiple determination (R^2) was 0.91 ($p<0.001$). Sixty-eight percent of the variation in disposal rate was predicted by plasma retinol, 18% by liver vitamin A and 14% by intake. These results imply that a pool of retinol which equilibrates with plasma retinol is a site of vitamin A degradation: as plasma retinol increases, so does degradation. When plasma retinol is at its homeostatic upper limit, further increases in vitamin A intake or stores can result in higher rates of vitamin A degradation.

Finally, high environmental temperature, as well as infection and other inflammatory responses, have been shown to lower plasma retinol and RBP levels, and also probably hepatic secretion of retinol. Thus we need to think about how these factors might affect the mobilization into plasma of our oral load of deuterated retinol. This would result in an overestimate in our calculation of total liver vitamin A reserves in the populations we will be studying. We will return to this point in the next section.

3. AREAS OF RESEARCH AND DEVELOPMENT THAT NEED TO BE CONSIDERED

In this section, I present issues related to the isotope dilution method that require future research and/or development. Some of these are important no matter what we eventually choose for an isotope dilution method; others relate directly to the methods of Bausch and Rietz [1], and Furr *et al.* [2], or to potential modifications of those techniques.

3.1. General issues

First, it will be critical to determine what the ideal stable isotope tracer will be for the proposed field studies. In view of the chemistry of our tracee, we will be considering ^{13}C versus ^{2}H. While the former isotope is more stable in the vitamin A molecule, the natural abundance of ^{13}C is much higher than that of ^{2}H, creating the potential for signal-to-noise problems in our analytical procedures. In addition, ^{13}C-labeled compounds are more expensive than those labeled with ^{2}H. If we choose ^{13}C, we must ascertain that an

ample supply of uniformly labeled vitamin A will be available. If we choose ^{2}H, we need to be vigilant in determining that the preparation is not contaminated with ^{3}H (i.e., radioactive tritium). Unfortunately, our choice of tracer may be influenced by factors of economics (^{13}C will be significantly more expensive) and availability (presumably, an ample supply of ^{2}H will be easier to find). Furthermore, if a second assessment of liver stores is planned, then a different isotope should be used (e.g., if tetradeuterated retinol is used for a first test, then the octadeuterated form would be suitable for the second assessment).

Second, it will be useful to examine alternative approaches to a single oral dose, with the aim of reducing the acute load of administered labeled material while increasing the isotope enrichment in plasma. Two approaches seem worth investigating: repeated oral doses given within ~24 h and daily oral doses during the "mixing" period.

Third, researchers will need to develop/refine the isotope dilution method for subjects of different ages and in various physiological states. For example, the ideal time for blood sampling is likely not the same under all conditions. Pilot studies should be planned on small numbers of representative subjects for this purpose. Such studies will also allow the analytical teams to provide input on the oral dose required to give adequate detection capabilities at the appropriate sampling time(s).

Fourth, method(s) must be developed to determine the efficiency of vitamin A- and carotenoid absorption, and carotenoid bioconversion, in various populations and from different food sources. Surprisingly, even in animal models, simple and reliable methods for measuring (pro)vitamin A absorption and carotenoid bioconversion have not been worked out, and thus influences of physiological state, age, food source and preparation methods have not been adequately studied.

Also it will be important to measure vitamin A utilization (disposal rate) in different populations and physiological states, since this will not only influence setting requirements but also the value of a in the equation of Furr *et al.* [2].

Finally as noted above, researchers need to examine effects of inflammation on plasma isotope dilution during the "mixing" period, in view of the findings that vitamin A metabolism is altered in this state such that application of the equation of Furr *et al.* may overestimate liver reserves of vitamin A [2].

3.2. Topics related to the methods of Bausch and Rietz [1] and Furr *et al.* [2]

In order to investigate the influence of estimated vitamin A stores on plasma isotope dilution, the isotope dilution test needs to be done first in subjects with low vitamin A status. Then the test should be repeated after increasing the subjects' supplemental vitamin A intake. I believe that Marjorie Haskell and Ken Brown (U.C. Davis) are already doing this type of study. A related research project should be to empirically determine the influence of variable vitamin A intake on the plasma isotope dilution value during the "mixing" period.

Attention to several more theoretical issues would improve the usefulness of the isotope dilution method for assessing vitamin A stores. First, it would be worthwhile to apply more sophisticated multiple regression techniques (such as constrained regression analysis) to defining the values for F, s and a in the equation of Furr *et al.* [2]. This approach would allow us to assign confidence intervals to these coefficients and to obtain confidence intervals (i.e., a range) for the estimates of stored vitamin A, rather than a single value. For this purpose, we may wish to consult in advance with investigators who have expertise in advanced regression methodologies.

Also, as noted above, one might apply the transformation described by Cobelli *et al.* [4] to plasma isotope enrichment data in order to account for the presence of pre-dosing plasma tracer and for the amount of tracee in the dose. I would also recommend that the analytical methods be set up so that we can estimate the fraction of an oral dose present in plasma at the time of sampling. This would require the availability of an appropriate internal standard, an estimate of plasma volume and an accurate determination of the amount of label administered. By determining the fraction of dose in plasma, one removes an added source of potential error (i.e., determining tracee in plasma). Further, such an approach may reduce the influence of dietary vitamin A intake during the isotope dilution period on the fraction of administered dose in plasma, since we would be determining the tracer levels in plasma, rather the tracer/tracee ratio (which is more influenced by tracee input from the diet).

3.3. Topics related to modified isotope dilution methods

Several areas of research/development come to mind as one reviews published modifications of the isotope dilution method as originally proposed for assessing vitamin A stores. We may be able to develop several measures of vitamin A stores from the results of a single isotope dilution study. Four methods in addition to the method of Furr *et al.* [2] come to mind. First, it would be useful to more carefully define isotope kinetics during the "mixing" period. By so doing, it is possible that we might find an earlier, more optimal sampling "window" that reflects liver vitamin A stores. Based on currently-available data from rat and human studies [9,15], I estimate that an ideal time might be at 3-4 d after isotope administration. It would be useful to then empirically derive equations relating vitamin A stores to tracer in plasma at 3-4 d as we have done in the rat [15]. If it is not feasible to collect a sufficient number of liver biopsies for this purpose, we could mathematically relate total traced mass of vitamin A (M_T) to tracer in plasma at various times; methods for calculating M_T are presented later.

Second, I propose that we apply slope analysis to data collected when the tracer "die-away" curve has reached its terminal slope. Normally an inverse relationship exists between slope (fractional catabolic rate of the system) and vitamin A stores. Determining the slope would also provide the information needed to calculate $\underline{a}$ in the equation of Furr *et al.* [2].

Another kinetic approach is available that allows us to determine a subject's total traced mass of vitamin A (M_T). It is potentially feasible to apply this technique in a small number of subjects in target populations. I will briefly describe the experimental approach we have used to estimate M_T in rats before describing the kinetic methods. I will conclude by mentioning modifications of these procedures that would be needed for application in humans.

We have used rats as an animal model for studying several aspects of vitamin A metabolism/assessment that seem relevant to these factors in humans. Obviously, with rats one has the luxury of controlled conditions with respect to genetics, dietary vitamin A etc., and the possibility for long-term, serial sampling of blood, excreta and tissues. Although such luxuries are never available in human experimentation, I firmly believe that a good animal model is the place for development of methods that can be modified to provide useful information in humans.

In order to measure M_T in rats, we administer an IV pulse dose of [^{3}H]retinol-labeled plasma to recipient rats. Labeled plasma is obtained from a vitamin A-deficient donor rat at ~100 min after administration of a dispersion of [^{3}H]retinol in Tween 40. We then sample the plasma of recipient rats for ~40 days, until the plasma [^{3}H] disappearance curve is well into a terminal slope (i.e., on a semilog scale, the

disappearance curve fits a straight line, allowing extrapolation to infinity). At early times, plasma is sampled frequently so that the shape of the plasma ^3H disappearance curve can be accurately defined; later blood samples are more widely spaced. In order to initially choose sampling times, it is useful to apply a geometric progression. More details on experimental design and methods have been published [19].

We have used two kinetic approaches to analyze our data on the fraction of injected isotope dose remaining in plasma versus time: model-based compartmental analysis and empirical analysis [12,20,21]. For model-based compartmental analysis, plasma data are fit to a 3- or 4-compartment model using CONSAM [16]. We calculate the plasma retinol pool size [M(1)] from the measured plasma retinol concentration and the estimated plasma volume. Then, assuming a steady state, we use CONSAM to estimate the mass of vitamin A in other compartments [M(I)] as well as many other interesting kinetic parameters, including disposal rate. Finally, the total traced mass M_T is estimated as the sum of the M(I)s.

In order to use empirical analysis to estimate M_T, data on fraction of dose in plasma versus time are fit to a 3- or 4-component exponential equation of the type:

$$y(T) = \Sigma\, I(i)*EXP[-g(i)*T]$$

where I(i) are the intercepts, g(i) are the slopes, EXP is the base e of the natural logarithm, and T is time. The sum of the intercepts [I(i)] is normalized as follows:

$$H(i) = I(i)/\Sigma\, I(i).$$

Then one calculates the mean sojourn time (MST) of labeled vitamin A as

$$MST = \Sigma\, (H(i)/g(i)^2)/\Sigma\, (H(i)/g(i)) = \int y(T)*TdT \,/\!/ \int y(T)dt$$

where $\int$ is the integral from time zero to infinity. The fractional catabolic rate $(FCR)_p$ of labeled vitamin A as

$$FCR_p = 1/T_p$$

where T_p is the residence time for the label in plasma and is computed as

$$T_p = AUC_p = \Sigma\, H(i)/g(i)$$

where AUC_p is the area under the plasma tracer response curve integrated from time zero to infinity. Then vitamin A utilization rate (disposal rate, DR) is computed as

$$DR = FCR_p * M_p$$

where M_p is the plasma retinol pool size [M(1) above]. Finally, total traced mass of vitamin A (M_T) is calculated as

$$M_T = DR * MST.$$

An estimate of M_T could be used to help validate/modify the equation of Furr *et al.* [2] in new populations under investigation in this project.

I mentioned above that, as liver stores of vitamin A get large, the vitamin may not "mix" with the absorbed labeled vitamin A in a reasonable period of time. For the rat, this problem is illustrated in Fig. 10 which shows data from all of the vitamin A turnover

studies conducted in my laboratory to date. As liver vitamin A begins to increase from a depleted state, the total traced mass is greater than liver vitamin A, indicating that extrahepatic stores of vitamin A exist. When liver vitamin A exceeds ~1000 nmol (~80 nmol/g), the total traced mass is less than the chemically-determined amount of vitamin A. Although some of the studies in vitamin A-adequate rats were carried out for 115 d, the tracer had still not mixed with these stores. These observations need to be kept in mind as we apply isotope dilution methods to human populations with widely differing stores of vitamin A in liver and whole body.

Lastly, I feel we need to reexamine the possibility that urine metabolites might be used as markers of vitamin A stores.

To conclude this section, I would like to flag the following features of the methods we have used in rats that would need to be modified to come up with a method that is feasible in humans. Clearly, we will need to administer the dose orally rather than intravenously, and we will need to use stable isotope-labeled vitamin A instead of radioactive retinol. Furthermore, we will need to have high enough tracer enrichment in the dose to follow plasma tracer disappearance for ~120 d. I base this estimate on the kinetic analysis I performed on the data of DeWitt Goodman (Fig. 4) on vitamin A turnover in 3 men [9]. Also, we must determine the minimum number of plasma samples and the optimal sampling times for obtaining an accurate portrait of the plasma isotope response curve. For this, one can apply sensitivity analysis [16]. In addition, it will be necessary to measure plasma retinol concentration at several selected sampling times, and to analyze a sufficient number of plasma samples collected during the period of dose absorption (e.g., 0.5 - 8 h post dosing) for labeled retinol versus retinyl esters. I also suggest that we use model-based compartmental analysis to analyze the data and calculate total traced mass of vitamin A rather than empirical analysis (multiexponential curve fitting). Finally, I feel that the experiments we are planning will be significantly better if we strive to keep involved, or recruit, scientists with expertise in isotope dilution methods (e.g., Harold Furr), as well as researchers who are experts in tracer kinetics, mathematical modeling and advanced regression methods.

4. ISOTOPE DILUTION ISSUES RELATED TO PREGNANCY AND LACTATION

There is no doubt that the physiological state of the field study subjects will complicate the application of isotope dilution methods to assess vitamin A status. One important fact that we will need to be mindful of is that pregnant/lactating women, and nursing children, are in a non-steady/variable state with respect to vitamin A. The biokinetic behavior of an orally administered stable isotope will be affected by the tracee's non-steady state.

Further, I predict that at least two features of normal vitamin A metabolism will be altered in pregnancy/lactation and thus need forethought. First, we need to have more information on the role of chylomicrons versus retinol-binding protein as alternative vehicles for delivery of vitamin A to fetus and milk. Second, it is likely that whole-body utilization of vitamin A will be influenced because of transfer of the vitamin to fetus and milk. These processes and their impact on the isotope dilution method need to be investigated. Finally, the choice of dosing regimen (a single large oral dose versus multiple smaller doses) needs to be considered in view of the possibility [22] that a large acute oral load has the potential to be teratogenic to the developing fetus. It is possible that chylomicron delivery of vitamin A, which will be proportional to dietary vitamin A intake, may be responsible for a less well-regulated delivery of vitamin A to the developing fetus.

5. CONCLUDING THOUGHTS

In summary, while some form of the isotope dilution method or a related kinetic evaluation is currently our best bet for a practical and accurate technique for assessing vitamin A status in the field, there are many important and challenging issues that need to be addressed, preferably before field studies are begun. I believe that it will be possible to meet these challenges and that, in so doing, we will have developed a technique for determining vitamin A status that has wide and useful application in humans.

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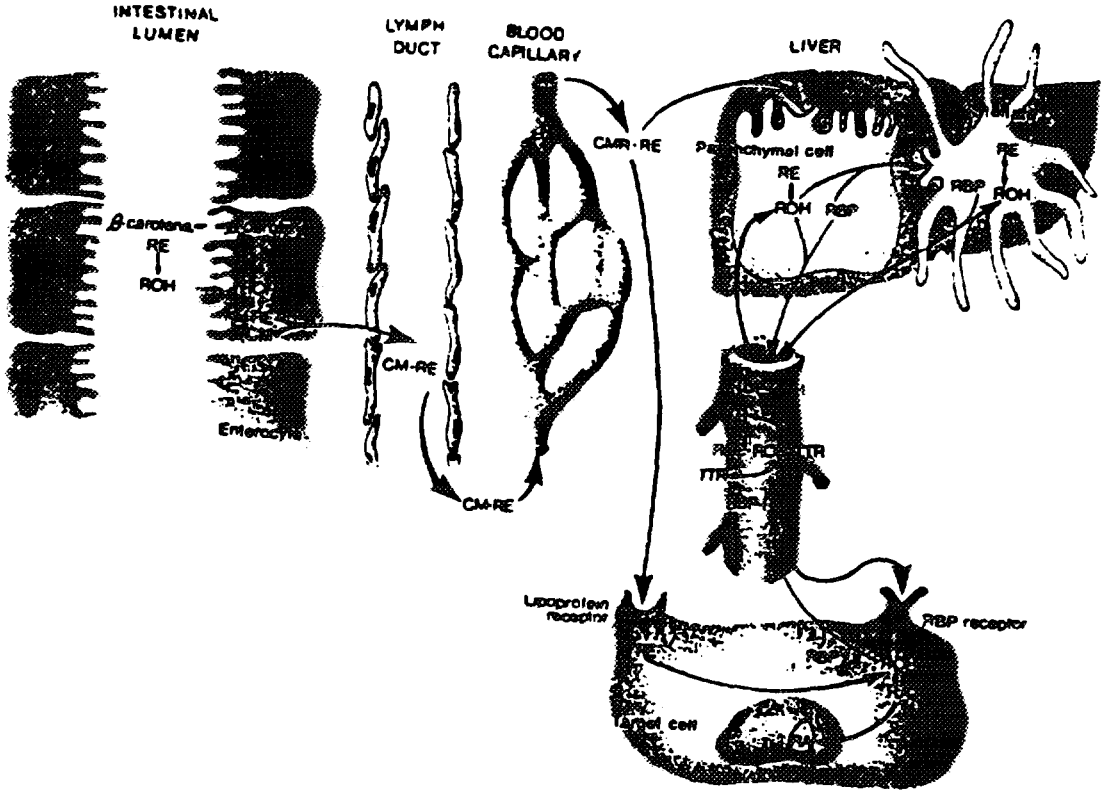

Fig. 1. Schematic of vitamin A metabolism as drawn by Blomhoff [8]. RE, retinyl esters; ROH, retinol; CM, chylomicron; CMR, chylomicron remnant; RBP, retinol-binding protein; TTR, transthyretin; RA, retinoic acid.

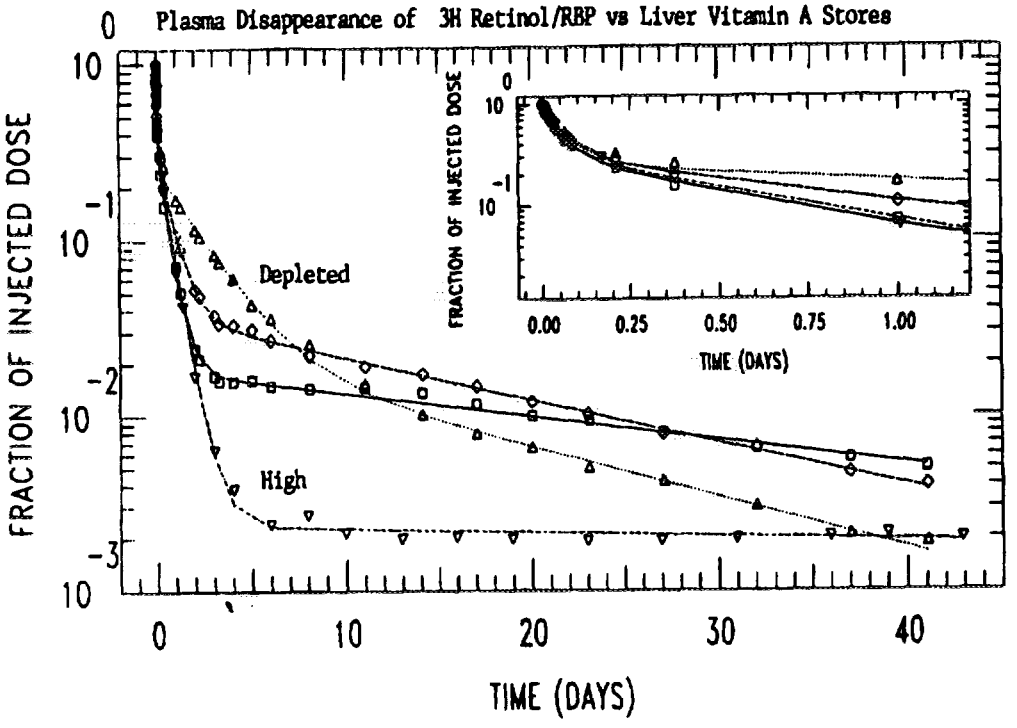

Fig. 2. Temporal disappearance of [^{3}H]retinol from plasma of rats at four levels of vitamin A nutriture. Rats had either high liver vitamin A stores (~9700 nmol) and consumed a low vitamin A-containing diet such that they were in a negative vitamin A balance (inverted triangles); or a liver vitamin A level of ~500 nmol at the time of administration of [^{3}H]retinol and an intake that resulted in slight positive vitamin A balance so that liver levels increased to ~670 nmol by the end of the 41-day turnover study (boxes); or a starting liver level of ~370 nmol that decreased, as a result of a low vitamin A intake, to ~44 nmol by the end of the study (diamonds); or an initially depleted level of liver vitamin A (~8 nmol) and low plasma retinol concentration (~2 versus ~1 μmol/L in the other groups) both of which increased by the end of the turnover study (to ~18 nmol in the liver) even though rats were consuming the same intake as those depicted by [diamonds]. Lines are fits to the data using a three- or four compartment model. Inset shows data for the first day. Data are from Refs. 12 and 13.

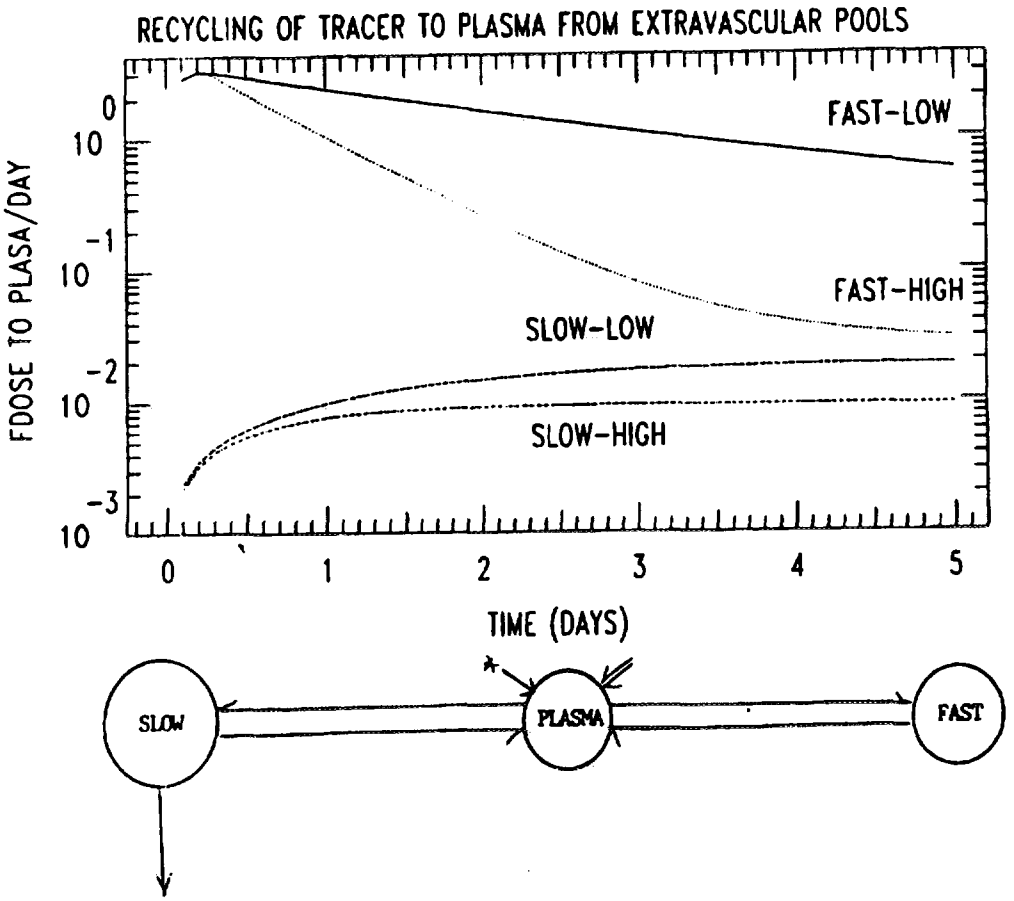

Fig. 3. Model-predicted recycling of [^{3}H]retinol to plasma from extravascular pools. The plasma disappearance data shown in Fig. 2 for rats with "depleted" or "high" liver vitamin A stores were fit to a 3-compartment model (lower panel). Recycling from the fast- and slow turning-over extravascular compartments was simulated (upper panel).

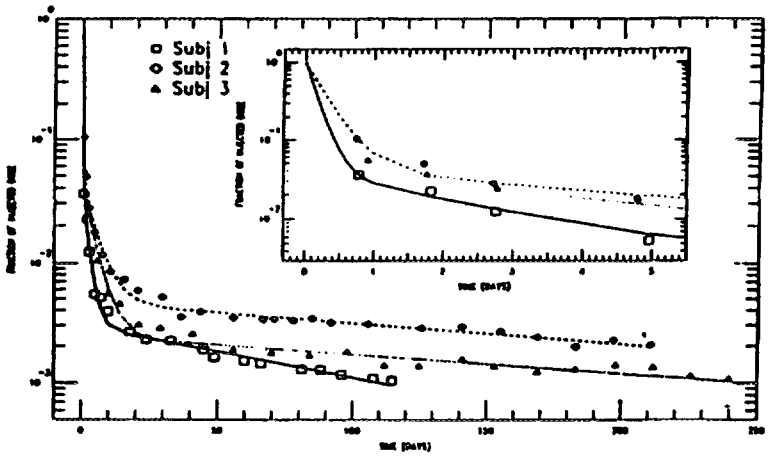

Fig. 4. Plasma retinol kinetics in humans. Shown are observed data (symbols) and model-predicted values (lines) for fraction of injected [^{14}C]retinol remaining in plasma versus time after dose administration. The model is shown in Fig. 5. This figure is reproduced from Ref. 9, Fig. 1.

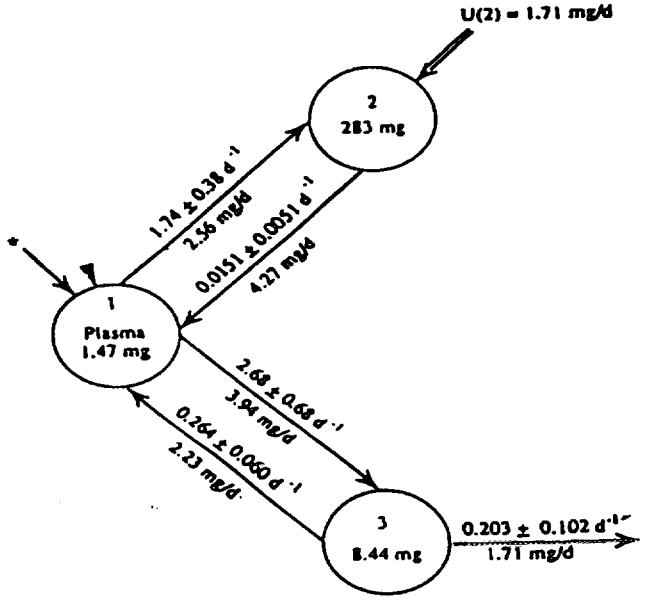

Fig. 5. Compartmental model for retinol kinetics in humans. Compartments are represented by circles; estimated compartment masses (mg) are shown inside each circle. Asterisk and stippled triangle show site of input of [^{14}C]retinol and site of sampling, respectively; U(2) is the estimated input rate for absorbed dietary vitamin A. Fractional transfer coefficients ± the population estimate of the standard deviation (d^{-1}) and transfer rates (mg/d) are shown above and below each arrow, respectively.This figure is reproduced from Ref. 9, Fig. 2.

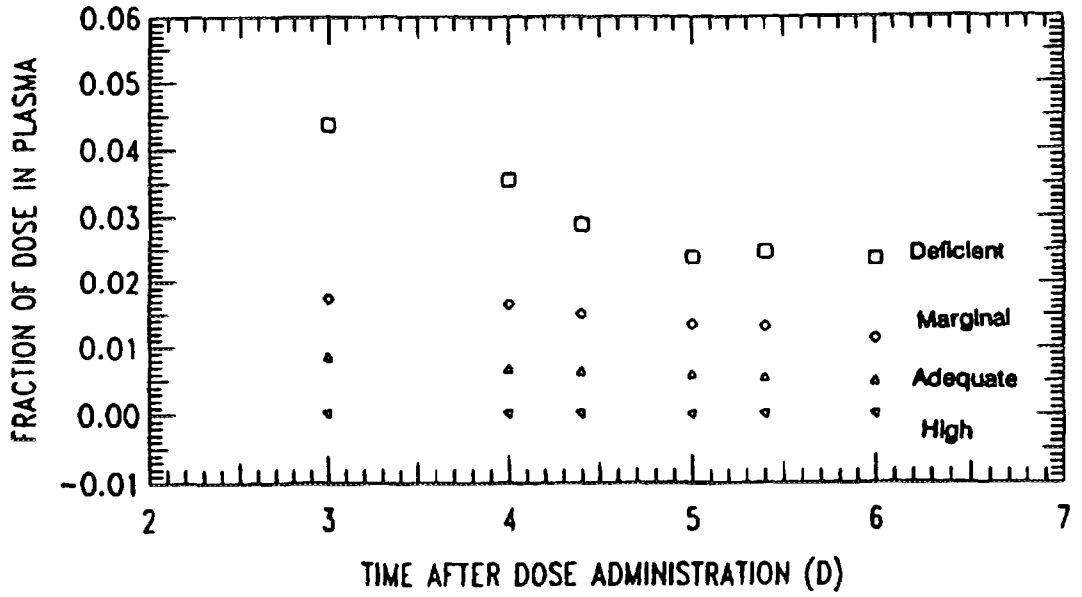

Fig. 6. Fraction of an oral dose of [^{3}H]retinol recovered in plasma versus time after dose administration for a randomly-selected rat from groups with deficient, marginal, adequate or high liver vitamin A levels. This figure is reproduced from Ref. 15, Fig. 1.

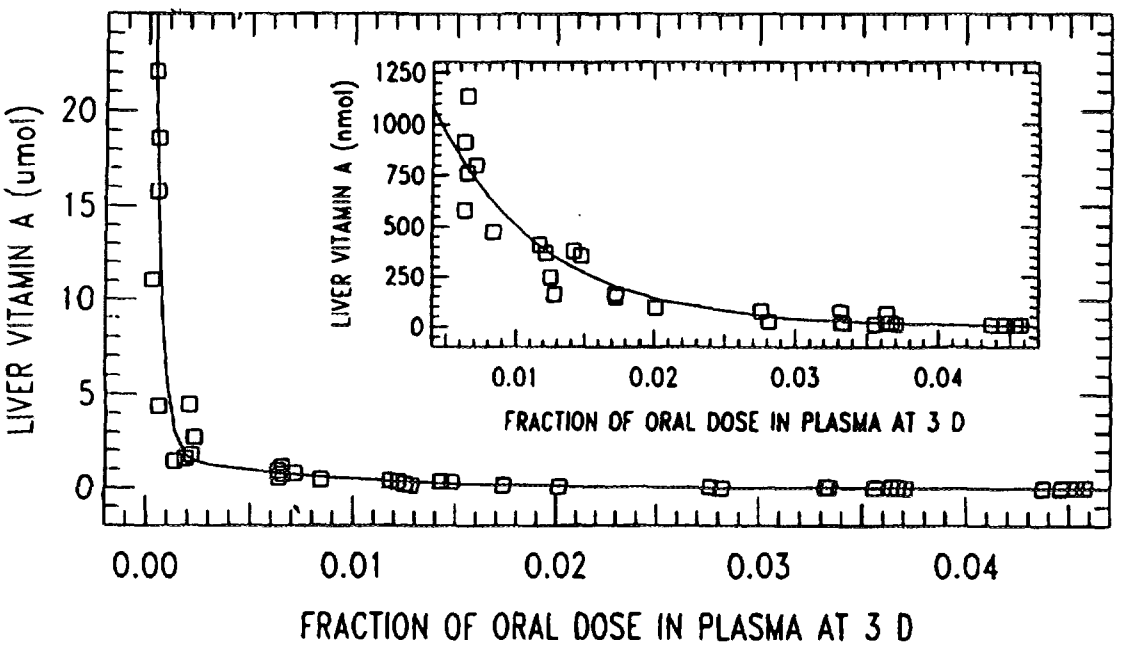

Fig. 7. Prediction of liver vitamin A from plasma retinol kinetics. Shown are observed values (squares) and predicted data (lines) for liver total retinol versus the fraction of an oral dose of [^{3}H]retinol in plasma at 3 days. This figure is reproduced from Ref. 15, Fig. 2.

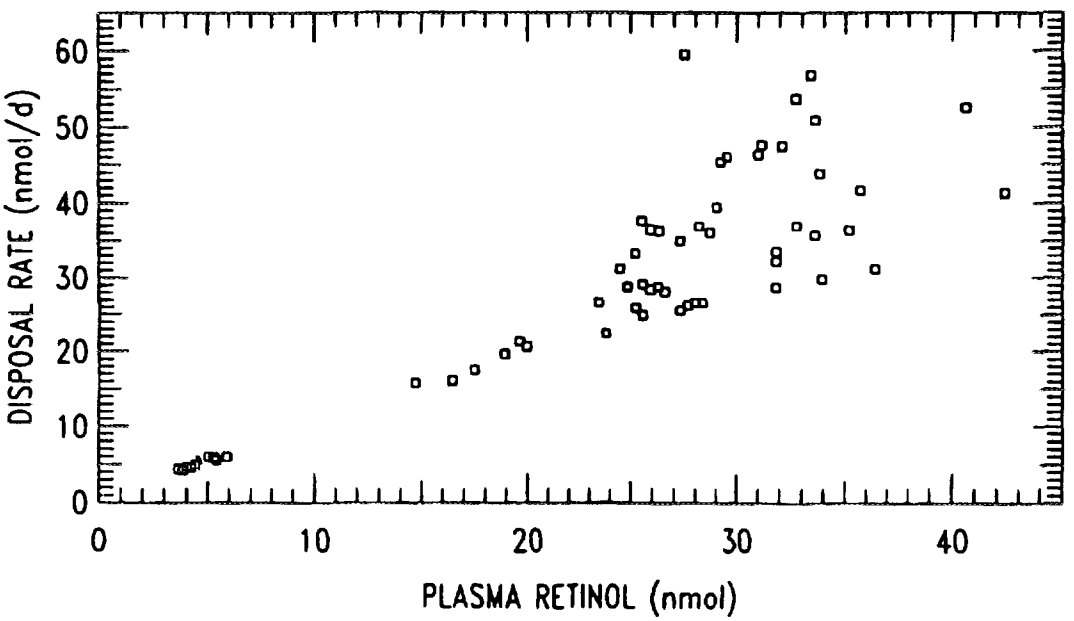

Fig. 8. Relationship between plasma retinol and disposal rate in rats with liver vitamin A levels ranging from 1 to 11,000 nmol. Data are from Ref. 13.

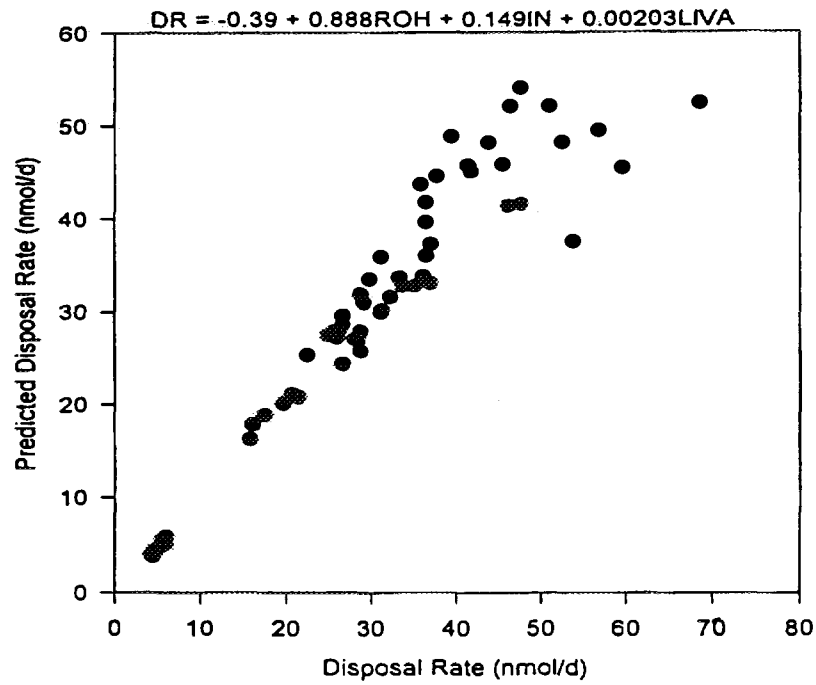

Fig. 9. Relationship between vitamin A disposal rate calculated from compartmental analysis of plasma [^{3}H]retinol disappearance data and that predicted from a multiple regression equation (see text) relating plasma retinol, vitamin A intake and liver vitamin A mass. Data are from Ref. 13.

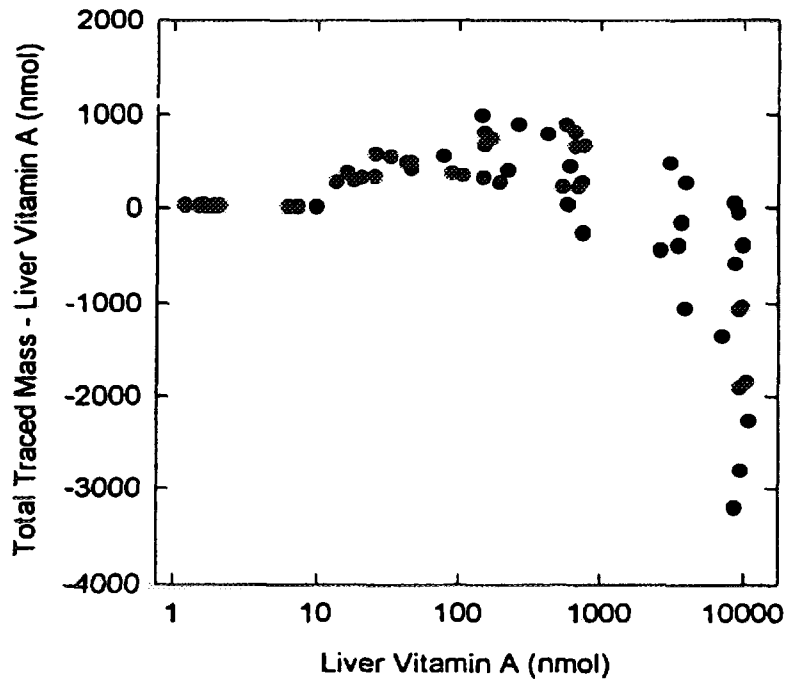

Fig. 10. Relationship between liver vitamin A and model-predicted total traced mass minus liver vitamin A. Data are from Ref. 13.