S-4-4 Ascorbic Acid and Reaction Kinetics *in situ*: A New Approach to Vitamin Requirements

M.LEVINE, K.R.DHARIWAL, P.WASHKO, R. WELCH,
Y-H WANG, C.C.CANTILENA, and R.YU

Laboratory of Cell Biology and Genetics, Building 8 Room 415,
The National Institutes of Health, Bethesda, MD 20892 USA

THE PROBLEM OF OPTIMAL VITAMIN REQUIREMENTS

Ascorbic acid (vitamin C) is required for human health (1-3). Most mammals synthesize ascorbic acid from glucose. However, humans and non-human primates do not produce gulonolactone oxidase, the terminal enzyme in the biosynthetic pathway (4). As a consequence, ascorbic acid needs must be met by dietary ingestion. Without ascorbic acid, the deficiency disease scurvy always occurs, and is fatal if untreated (1).

How much ascorbic acid do humans need? The selection of 60 mg daily, the recommended dietary allowance (RDA) in many countries, is based on several observations. One is from clinical studies, in which approximately 10-12 mg of ascorbic acid daily prevented scurvy (2,3). Thus, an RDA of 60 mg provides a wide margin of safety against scurvy (2). A second observation is that ascorbic acid is excreted in urine once plasma concentrations reach a threshold of approximately 30 μ M (2,3). With respect to requirements, the concept is simply that when vitamin excretion occurs, higher amounts in plasma are unnecessary (2). Since ascorbic acid urinary excretion occurs when ingestion is more than 60 mg daily, 60 mg was a logical choice as the RDA. A third observation is that the body pool size of ascorbic acid is approximately 1500 mg, and that this can be achieved by 60 mg per day of ascorbic acid (2).

There are several problems with these basic concepts. Ingestion of ascorbic acid at the RDA amount clearly prevents scurvy. But prevention of scurvy may not be the same as providing an optimal amount of ascorbic acid for human health (3,5). Likewise, urinary excretion of the vitamin may not be an adequate index of vitamin requirements. For some substances such as glucose, urinary excretion is an indication of excess. But for other substances, such as sodium and potassium, urinary excretion has no relationship to tissue saturation. The assumption that the kidney is a barometer for vitamin requirements is at best naive, and more likely is incorrect (3,5). Similarly, body pool measurements of ascorbic acid are based on assumptions of ascorbic acid metabolism which may be correct for ingestion of very small amounts of ascorbic acid but not for higher ones. A consequence of these incorrect assumptions is underestimation of the ascorbic acid body pool.

The RDA would seem reasonable if similar amounts of ascorbic acid were available in the diet. However, a diet rich in fruits and vegetables provides at least ten fold more ascorbic acid than

the RDA. In one recent example, our paleolithic ancestors were estimated to consume a diet containing 6-7 fold more ascorbic acid than the RDA (6). The RDA would also seem reasonable if ascorbic acid toxicity were an issue, or there was only a small margin of safety between preventing deficiency and toxicity. However, ascorbic acid is remarkably non-toxic (3), which might be predicted given its wide availability in fruits and vegetables. Another indication of whether the RDA is correct comes from ascorbic acid biosynthetic rates in many animals. On a weight adjusted basis, animals that synthesize ascorbic acid are estimated to make 10-100 times more than the human RDA. These calculations are far from ideal; nevertheless, they suggest that the current RDA is too low to represent optimal intake (3). Another clue comes from primate experiments; primates maintained in captivity require 10-20 fold more ascorbic acid than the RDA on a weight adjusted basis (3).

The RDA provides for minimal ascorbic acid amounts, but not for optimal ingestion. What, then, are assays for optimal ascorbic acid requirements? Unfortunately, until recently there have been no reliable assays for optimal requirements for ascorbic acid, or in fact for other vitamins (3,5). Simply stated, the problem is that the minimal may not equal optimal, but there has been no way to measure the difference.

IN SITU KINETICS

We have proposed and recently validated the first solution to the problem of optimal vitamin requirements; the approach is called in situ kinetics. The principles of in situ kinetics were developed using ascorbic acid as a model vitamin, but the principles are applicable to all vitamins. In situ kinetics provides a new approach for determining ideal vitamin requirements by quantitating how vitamin concentration regulates function. Biochemically, this is the application of Michaelis-Menten reaction kinetic principles to vitamin dependent reactions in situ. Surprisingly, such an approach has never been proposed prior to the work of this laboratory. Ascorbic acid is required for activity of eight isolated enzymes, and may also be a part of other non-enzymatic reactions (3,5). To determine optimal vitamin requirements, we must measure how different vitamin concentrations specifically regulate these reactions. Rather than just using isolated proteins, the reactions must be studied in situ in their appropriate physiological milieu (i.e. in cells, organelles, and tissues) (3,5).

In situ kinetics also has a clinical component. A key issue is to determine how dietary ingestion of ascorbic acid regulates the circulating amount of vitamin that is available to tissues, so that the biochemical reactions can occur (7). Using in situ kinetics, ideal vitamin requirements will be learned by measuring how variations in circulating ascorbic acid occur in relation to intake, how these variations control intracellular vitamin concentration, and how intracellular vitamin concentration in turn regulates specific vitamin-dependent biochemical functions.

There are at least eleven specific components of in situ kinetics:

- * Convenient access to a mammalian cell/organelle/tissue that contains the vitamin;
- * A known or possible enzymatic/non-enzymatic function of the vitamin in this tissue;
- * Characterization of the vitamin's intracellular distribution;
- * Co-localization of the vitamin and the reaction in which it participates, or an explanation for lack of co-localization;
- * Ability to regulate intracellular/subcellular vitamin concentration across a wide range; an understanding of vitamin transport, depletion, and repletion;
- * A very sensitive, specific, and reproducible method to measure the vitamin dependent reaction in situ;
- * Specificity of the vitamin for the reaction; involvement of the vitamin at one or multiple steps in a reaction sequence;
- * Potential physiologic importance of the reaction;
- * Measurement of the reaction in situ as a function of intracellular and extracellular vitamin concentration;
- * Determination in humans of vitamin availability to tissues, to allow cell specific reactions to occur; determination of how ingestion regulates extracellular and intracellular vitamin concentration.

The components of in situ kinetics were derived from our experimental work (7-13), and are not rigid. We anticipate that new ideas will be added as other in situ systems are studied.

MODEL SYSTEMS FOR IN SITU KINETICS

In situ kinetics can be theoretically represented by dose response curves, where each curve displays the amount of an ascorbate-mediated biochemical event in relation to ascorbate concentration (3,5). The curves are equivalent to substrate velocity plots. Toxicity would be indicated by a simple fall in activity. Based on the shape of the curves, a concentration of ascorbic acid should be achieved that produces maximum in situ activities without toxicity (5).

These curves are more than theoretical; they can be achieved using in situ systems. As our first model system, we used ascorbic acid dependent norepinephrine biosynthesis in animal tissue, mediated by the enzyme dopamine beta-monooxygenase. These studies showed that norepinephrine biosynthesis was dependent on ascorbic acid in cells and isolated secretory vesicles (8,9). Ascorbic acid action in situ was more complex than its interaction with the isolated enzyme (8-11). Ascorbic acid action in situ was two fold: first, as a true co-substrate for the intra-vesicular enzyme dopamine beta-monooxygenase (10); second, for regeneration of intragranular ascorbic acid via electron transfer across the secretory vesicle membrane (8,10,11).

These data provided the first evidence that in situ kinetics can be achieved for any vitamin by using an intact physiologic system with the endogenous vitamin and substrate. These experiments indicated that in situ systems must be used as well as endogenous substrate and endogenous vitamin. These data also show that the two vitamin dependent reactions normally proceed at

$V_{\max}$ in situ. These observations provide a firm foundation for using reaction rates to determine optimal requirements.

The principles of in situ kinetics are now being studied in human systems in our laboratory, including human neutrophils (12), human lymphocytes (13), and human fibroblasts (manuscript submitted). Experiments are also underway in human subjects to determine ascorbic acid availability to tissues (7).

SUMMARY

Biochemical and clinical evidence indicate that in situ kinetics is the first quantitative and achievable approach for determining optimal requirements for ascorbic acid, and for other vitamins.

REFERENCES

1. Crandon J.H., Lund C.C., and Dill D.B. (1940) Experimental human scurvy. *N Eng J Med* 223:353-369.
2. Food and Nutrition Board. Recommended Dietary Allowances. Ninth ed, Washington D.C. National Academy Press 1980:72-82. Water soluble vitamins: vitamin C.
3. Levine M. (1986) New concepts in the biology and biochemistry of ascorbic acid. *New England Journal of Medicine* 314:892-902.
4. Nishikimi M. and Udenfriend S. (1976) Immunologic evidence that the gene for L-gulonolactone oxidase is not expressed in animals subject to scurvy. *Proc. Natl Acad. Sci. USA* 73: 2066-68.
5. Levine M. and Hartzell W.O. (1987) Ascorbic acid: the concept of optimum requirements. *Ann. N.Y. Acad. Sci.* 498:424-44.
6. Eaton S.B., Konner M. (1985) Paleolithic nutrition. *N. Eng. J. Med.* 312:282-289.
7. Dhariwal K., Hartzell W., and Levine M. (1991) Measurement of ascorbic acid and dehydroascorbic acid in human plasma and serum. *Amer.J.Clin.Nutr.* In press.
8. Levine M., Morita K., Heldman E., and Pollard H.B. (1985) Ascorbic acid regulation of norepinephrine biosynthesis in isolated chromaffin granules from bovine adrenal medulla. *J. Biol. Chem.* 260:15598-15603.
9. Levine M. (1986) Ascorbic acid specifically enhances dopamine beta-monooxygenase activity in resting and stimulated chromaffin cells. *J. Biol. Chem.* 261:7347-7356.
10. Dhariwal K.R., Washko P, Hartzell W.O., and Levine M. (1989) Ascorbic acid within chromaffin granules: in situ kinetics of norepinephrine biosynthesis. *J. Biol. Chem.* 264:15404-15409.
11. Dhariwal K. Shirvan M., and Levine M. (1991) Ascorbic acid regeneration within chromaffin secretory vesicles: in situ kinetics. *J. Biol. Chem.* 266:5384-87.
12. Washko P., Rotrosen D., and Levine M. (1989) Ascorbic acid transport and accumulation in human neutrophils. *J. Biol. Chem.* 264:18996-19002.
13. Bergsten P., Amitai G., Kehrl J., Dhariwal K., Klein H., and Levine M. (1990) Millimolar concentrations of vitamin C in purified mononuclear leukocytes: depletion and reaccumulation. *J. Biol. Chem.* 265:2584-2587.