

# Proceedings of the XX International Vitamin A Consultative Group Meeting

## Physiologic Indicators of Vitamin A Status<sup>1,2</sup>

Nathan G. Congdon\*<sup>3</sup> and Keith P. West, Jr.<sup>†</sup>

\*The Dana Center for Preventive Ophthalmology, The Johns Hopkins University Schools of Medicine and Public Health, Baltimore, Maryland and <sup>†</sup>Program in Nutrition, Department of International Health, The Johns Hopkins University School of Public Health, Baltimore, Maryland

**ABSTRACT** Physiologic indicators reflect the functional consequences of vitamin A deficiency and may be particularly useful for detecting early perturbations in vitamin A status. In conjunctival impression cytology (CIC), epithelial morphology and the presence or absence of mucin spots and goblet cells allow samples, obtained by applying filter paper to the temporal conjunctiva, to be characterized as normal or typical of vitamin A-deficient keratinizing metaplasia. The validity of CIC has been established with reference to other indicators of vitamin A status, and a prevalence of  $\geq 20\%$  abnormal results has been suggested as indicative of a public health problem. However, interpretation of specimens requires considerable training, and nonresponsiveness to supplementation is a frequent problem, which limits the utility of CIC as a method for evaluating the impact of intervention programs. Several simplified field protocols for dark adaptation have been developed, including one in which dark adaptation is assessed by the responsiveness of the pupil to light. Night blind subjects have consistently shown abnormal results on this test, and a significant response to placebo-controlled dosing has been demonstrated for children and pregnant women. Scores have correlated significantly with serum retinol and relative dose response. Pupillary dark adaptation testing is acceptable to most children as young as 2 y old. Limitations of this technique include a time course for recovery after dosing as long as 4–6 wk, a testing time of 20 min, and the need for 1–3 d of training. Given its low cost, noninvasive nature, and lack of the need to transport samples, pupillary dark adaptation offers advantages over other techniques for assessing a population's vitamin A status. J. Nutr. 132: 2889S–2894S, 2002.

**KEY WORDS:** • *physiologic indicator* • *conjunctival impression cytology* • *dark adaptation* • *vitamin A* • *pupil*

Physiologic indicators are intended to detect mild or early functional disturbances in the body that are specific to vitamin A deficiency. As such, they are intended to reflect early manifestations of deficiency and, ideally, respond to vitamin A repletion. Mild pathophysiologic change may occur in parallel with or may follow measurable depletion of vitamin in circulation or in tissues.

The role of vitamin A in retinal function provides a classic example of a physiologic target. Depletion of vitamin A induces early disturbances in dark adaptation that can be detected by noninvasive testing before night blindness (XN)<sup>4</sup> is clinically evident. As a result, much work has been done to develop indicators to detect early visual disturbances through dark adaptometry before clinical or behavioral recognition of XN.

Vitamin A is known to regulate cellular differentiation, which is highly responsive to changes in vitamin A nutriture among rapidly dividing epithelial, osteoid and immune-competent cells. Indicators based on the measurement of status or change in such cell systems may be expected to reflect vitamin A status. Practicality in terms of access, and concerns about the specificity of the relationship, have generally led investigations to assess epithelial tissues, and especially the ocular surface through impression cytology (1), as a means to observe functional changes in vitamin A nutriture and assess popula-

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<sup>3</sup> To whom correspondence should be addressed.  
E-mail: ncongdon@jhmi.edu.

<sup>4</sup> Abbreviations used: CIC, conjunctival impression cytology; ICT, impression cytology with transfer; IU, international unit; NNIPS-2, Nepal Nutrition Intervention Project Sarlahi-2; RDR, relative dose response; RE, retinol equivalent; XB, Bitot spots; XN, night blindness.

tion status. Buccal mucosal (2,3) and vaginocervical epithelium (1,4) assays have received limited attention.

The role of vitamin A in regulating nitrogen retention and amino acid metabolism has prompted the exploration of changes in plasma and urinary indicators of protein metabolism, such as the measurement of urea and ammonium nitrogen, in relation to conventional indicators of vitamin A status (5,6).

Physiologic indicators are often evaluated in comparison with one or more conventional indicators such as clinical signs and symptoms, plasma retinol concentration and relative liver adequacy of vitamin A [e.g., relative dose response (RDR) or its modification] (7–11). Future studies can be expected to validate indicators against stable isotopically derived estimates of total body vitamin A stores (12).

Among the physiologic indicators of vitamin A status currently available, only impression cytology and dark adaptometry have received sufficient attention to evaluate their performance as practical tools for population assessment of vitamin A-dependent function.

## CONJUNCTIVAL EPITHELIAL DIFFERENTIATION

### *Impression cytology*

The corneal and conjunctival epithelia undergo keratinizing metaplasia in the vitamin A-deficient state, as do epithelial cells in other organs (13). Histologic abnormalities in the bulbar conjunctiva, including separation and distortion of epithelial cells and losses of mucin-secreting goblet cells, occur with mild vitamin A deficiency before the onset of clinically apparent surface changes (14). Conjunctival impression cytology (CIC) and impression cytology with transfer (ICT), a modification of the initial method, are based on well-described histopathologic changes due to vitamin A deficiency.

A great deal of experience has been gained with impression cytology since it was first introduced as an indicator of ocular surface disruption (15) and vitamin A deficiency (16,17) in the mid-1980s. Briefly, the method involves gently applying a strip (17) or disc (18) of cellulose acetate filter paper to the temporal bulbar conjunctiva for 2–3 s, which, after removal, is placed in fixative. In the laboratory, filter paper specimens are stained, clarified, and mounted for examination. The ICT method differs in that, once collected, specimens are immediately transferred to a glass slide through finger pressure; they are then fixed and stained for viewing (19,20). Specimens are microscopically examined for the presence and density of goblet cells or mucin spots and the density, size, and shape of epithelial cells in relation to specimen area or number of microscopic fields (17,19,21). Abnormal specimens are often devoid of goblet cells and mucin droplets and are often characterized by large, separated or keratinized epithelial cells. However, a number of interim stages can be observed and substantial variation may exist within and between microscopic fields and eyes, leading to differences in diagnostic criteria and the potential for considerable interobserver error (17,19–26). The presence of a minimum density of goblet cells, with or without a normal epithelial sheet, typically defines a lower limit for a normal diagnosis. A “cutoff” of at least one versus both eyes being abnormal can be expected to increase sensitivity, with a greater proportion of deficient individuals detected leading to an increased prevalence, and possibly also improve indicator responsiveness to treatment (27).

### *Correlation of impression cytology with other indices of vitamin A status in an individual*

The validity of impression cytology is most apparent when its results are compared with composite indices of vitamin A deficiency. In an early study of 146 Indonesian preschool children, the frequency of CIC abnormality increased with the severity of deficiency based on the joint distribution of eye signs, their responsiveness to vitamin A treatment and serum retinol concentration (21). CIC correctly identified 93% of all definite cases and 94% of all definitely normal children. Values of 82% and 70% were reported in 236 Indonesian preschoolers by using a combined clinical signs plus serum retinol index as the gold standard (18). Sensitivity declines and specificity improves when serum retinol or RDR cutoffs are also used in the definition of a case (23,25,28,29). The use of one or both eyes in the case definition will also affect estimates.

The correlation between CIC and other indices of vitamin A status is apparent by the typically increasing percentage of abnormal subjects observed with decrements in serum retinol (18,22,27,30–32), increments in the RDR (31,33) and increases in the severity of xerophthalmic signs (17–18,21,34). Abnormal CIC/ICT has also been associated with health risks, albeit less strongly, that have been observed with mild xerophthalmia, such as anemia, otitis media, respiratory infection (34,35), persistent diarrhea (33) and mild anthropometric deficit (20,34,36–38), although not always (30).

### *Prevalence estimates of vitamin A deficiency by impression cytology*

Estimates of the prevalence of vitamin A deficiency by impression cytology in children are frequently 5–10 times the rate of xerophthalmia, from 20 to 65% (18,20,25,30,32,34–37,39–42). These ranges are comparable to those reported by the World Health Organization from community-based, serum retinol surveys conducted among child populations in these regions over the past few decades (43). Limited data among pregnant and lactating women in South (28) and Southeast (27) Asia suggest an abnormal CIC prevalence of ~30%, also comparable to serological data (44–47).

Experience indicates that impression cytology is a feasible (48,49) and reliable indicator of vitamin A status that is appropriate for survey use. A prevalence of  $\geq 20\%$  has been suggested as a provisional cutoff for reflecting a vitamin A deficiency problem of public health significance (50), a figure equally applicable to women and children. However, a number of programs have found it difficult to interpret the specimens, even after considerable formal training, which is essential for obtaining reliable results.

### *Responsiveness of impression cytology*

Impression cytologic status responds to vitamin A intervention, although not always completely or predictably. Approximately 75% of Thai school-aged children participating in a randomized trial returned to normal status after daily vitamin A supplementation [1500  $\mu\text{g}$  of retinol equivalents (RE)/d] for 6 mo. There was a 100% response in people receiving vitamin A plus zinc (25 mg/d) (26). Among Indonesian women receiving 300,000 international units (IU) of vitamin A postpartum, the prevalence of abnormal CIC (at least one eye abnormal) was reduced by ~30 and 60% over a 3- and 6-mo period, respectively, compared with a relative decrease of only 15% by 6 mo observed among controls (27). In Senegal, where two thirds of 220 undernourished preschool children were ICT abnormal, only 46% returned to normal 2 mo after high-

potency vitamin A dosing, a period that included the peak mango season (32). A second Senegalese study also observed only ~50% of over 500 ICT abnormal children 2–15 y of age returning to normal 7 wk after they received 200,000 IU as either vitamin A or  $\beta$ -carotene (39). In Nepal, where ~20% of 472 children 24–60 mo of age were CIC abnormal, high-potency vitamin A dosing for 16 mo was associated with a cure rate of 82% versus 67% among CIC abnormal placebo recipients followed over the same period ( $P < 0.001$ ) (S. K. Khattri, Nepal Eye Hospital, unpublished data).

Nonresponsiveness of CIC or ICT to vitamin A supplementation may occur for several reasons. The period required for epithelial recovery after vitamin A receipt may extend, for many children, beyond 2 mo, as suggested by response rates in Senegal versus Thailand and Indonesia, where subjects were evaluated after 6 mo. Nepalese data suggest that over an even longer duration abnormal CIC may normalize through gradual changes in diet and other risk factors that may occur over time, or at least seasonally. Limitations on epithelial response to vitamin A may be imposed by concurrent zinc (26) or possibly folic acid (51–52) deficiencies, other nutritional disorders, diseases and environmental exposures. Ocular infection has been observed to mask conjunctival disturbance due to inflammation, increased blood flow and mucin production (53), which theoretically could mask an abnormal impression (17,25), artifactually lower the prevalence and mask a tissue response to vitamin A. However, the opposite has been observed, with higher abnormality rates reported in Indonesian (18) and Malian (40) children with conjunctivitis and inflammatory trachoma, respectively. It is not clear how intercurrent ocular infection may perturb the response of CIC/ICT to vitamin A supplementation over time. Studies in Bihar (54) of the natural history of Bitot spots (XB), which are clinical manifestations of abnormal conjunctiva, suggest that a significant number of children with vitamin A deficiency develop irreversible, localized metaplasia that can account for resistant, nonresponsive lesions.

Impression cytology provides a responsive physiologic indicator to changes in vitamin A intake; however, currently a variety of factors limit its utility as a means of assessing the effect of intervention programs.

## DARK ADAPTATION

### *Field techniques to assess dark adaptation*

Unlike CIC, no single agreed-upon protocol for carrying out dark adaptation under field conditions exists. Only those protocols for which substantial field data are available are addressed directly in this report.

It has been demonstrated that a maternal history of XN can be an accurate indicator of XN and impaired vitamin A status in a child (55). Where local terms exist, an oral history can thus be of great benefit in the population assessment of vitamin A status. However, local terms are not available for all populations, and dark adaptation testing has suggested that deficient persons may have measurably abnormal thresholds and yet not complain of clinical XN (56). Conventional, laboratory-based testing of dark adaptation usually requires at least 30 min, the period for a fully light-adapted eye to become completely dark-adapted. During this process, the eye becomes more sensitive to low light levels, in large part because of a shift from photopic vision, based on the cones, to scotopic vision, based on the rods. A number of rapid dark adaptation tests have been described (26,57–60). Usually taking 2–3 min, these tests have required subjects to identify a letter (26) or to

sort discs of different colors (58). A problem in common with all these rapid tests is that measurements carried out during the first few minutes of dark adaptation rely principally on the cones or light vision cells in the retina instead of the rods or dark vision cells, which has led to conflicting results (26,59–61) and makes such tests inherently less sensitive as indicators of vitamin A deficiency (62).

Practical field-based tests of vitamin A status should be reliable when used among preschool-aged children, the group most at risk for vitamin A deficiency. Traditional psychophysical dark adaptation testing, including the rapid methods discussed above, are not well suited to testing young children. Duncan et al. (63) recently reported a dark adaptation test that required young subjects (aged 6–67 mo) to follow a dim light projected on a wall after 10 min of dark adaptation, with movement of the head tracked by means of head-mounted illumination. Although dark adaptation thresholds measured in this way did not correlate significantly with serum retinol for all subjects, the correlation was significant ( $P < 0.05$ ) for subjects with serum retinol  $<0.35 \mu\text{mol/L}$ . Although dosing with vitamin A was carried out, results of postdose testing were not reported. Subjects were severely malnourished children admitted to the nutrition ward at a hospital in Kampala, Uganda.

### *Pupillary dark adaptation*

We have reported (56,64) on a completely different technique to assess dark adaptation among individuals of all ages, which requires no response and minimal cooperation from the subject. Our testing protocol uses the pupillary response as an indication of the subject's dark adaptation threshold. A portable, battery-powered apparatus connected to a hand-held illuminator is placed over the subject's left eye, and the light is incremented over 11 settings (roughly a 4-log unit range) until a pupillary response in the uncovered right eye is observed. This normal tendency of the pupil in one eye to respond equally to a stimulus in the contralateral eye is called the consensual response. The uncovered eye is examined with a  $\times 2.5$  visor-style loupe by means of an obliquely mounted red light, which preserves dark adaptation in both the subject and the observer. All testing is done after a partial bleach of the full retina (carried out by a standard camera flash reflected through a foil-lined cone), followed by 10 min of dark adaptation.

### *Validity of pupillary dark adaptation*

More data are available under field conditions for more populations with regard to the pupillary testing protocol than for other field techniques of dark adaptation testing (56,64,65). Interobserver reliability to within a single unit on the 11-unit scale has been demonstrated (56). Validation of the technique has been accomplished in several ways. Among populations of night blind pregnant women tested by Christian (28) (mean  $-0.84 \log \text{cd/m}^2$  for 77 women), L. Pizzarello (unpublished dissertation) (mean  $-0.91 \log \text{cd/m}^2$  for 67 women) and M. Haskell (University of California at Davis; unpublished data) (mean  $-0.70 \log \text{cd/m}^2$  for 88 women), all had mean thresholds significantly worse than for pregnant Nepali women in the Nepal Nutrition Intervention Project Sarlahi-2 (NNIPS-2) trial receiving either placebo ( $-1.11 \pm 0.39 \log \text{cd/m}^2$ ) or vitamin A ( $1.24 \pm 0.36 \log \text{cd/m}^2$ ) (45) (the more negative the score, the more sensitive the retina and therefore the better the subject's dark adaptation). Pregnant Nepali women also showed significantly worse scores during

the third trimester than during the first trimester (65), which correlates with increased reports of XN later in pregnancy (66). Second, a significant response to controlled dosing with vitamin A has been demonstrated for children (56) and pregnant women (65). Finally, dark adaptation scores have correlated significantly with serum retinol (56,64,65) and RDR (56).

This technique is meant to distinguish between normal and deficient populations. The various populations that have been tested to date can be divided into three groups: 1) normal, 2) untreated vitamin A deficient and 3) treated vitamin A deficient or unselected groups from deficient areas. As expected, the dark adaptation scores for the normal groups are best, followed by those from treated vitamin A-deficient subjects, with the scores of untreated vitamin A-deficient subjects being the worst (Fig. 1). When dark adaptation scores are plotted against serum retinol for those populations in which both have been measured, the relationship is very nearly linear (Fig. 2).

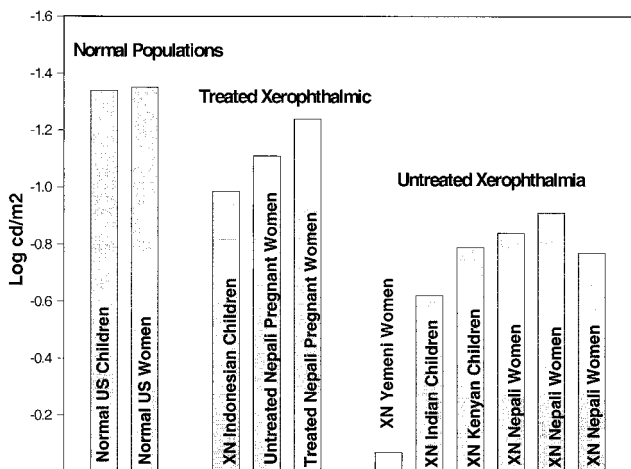
**Acceptability of pupillary dark adaptation**

Among women of reproductive age, ability to comply with testing under our protocol is in the range of 90% (65). Similar rates can be achieved with children aged 3 y and above. Even among the very youngest postweaning children, those at greatest risk for vitamin A deficiency, rates of compliance with pupillary testing are significantly higher than for traditional, psychophysical testing of dark adaptation (Fig. 3).

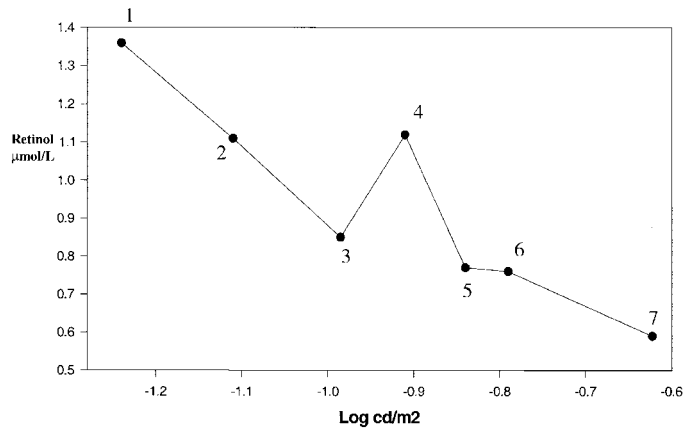
**Suggested cutoffs for intervention with pupillary dark adaptation**

Currently available information from a number of populations suggests that pupillary dark adaptation testing can provide a moderately high level of certainty that vitamin A deficiency is or is not a problem in an area. On the basis of present information, two cutoffs may prove useful to policy planners; both are based on the mean pupillary dark adaptation score for a population. Scores worse than the clearly deficient cutoff of  $-1.11 \log \text{cd/m}^2$  would be taken as evidence of vitamin A deficiency. Scores better than the clearly normal cutoff of  $-1.24 \log \text{cd/m}^2$  would suggest that a population is normal with respect to vitamin A status or that an intervention program has been successful in improving status.

The score used to define the clearly deficient cutoff was observed among unselected pregnant Nepali women receiving



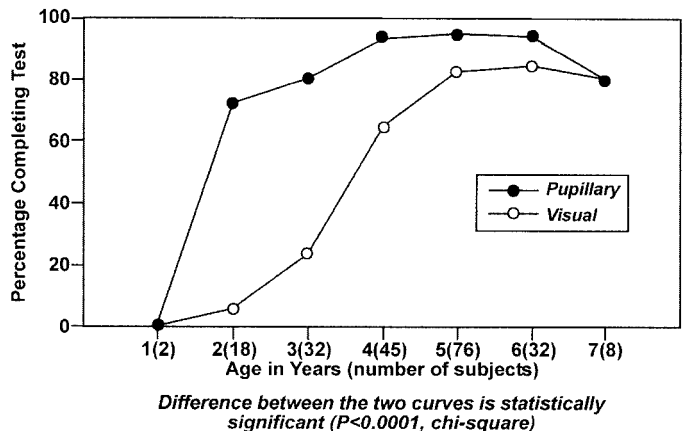
**FIGURE 1** Mean dark adaptation scores for normal, treated xerophthalmic and untreated xerophthalmic population groups.



**FIGURE 2** Serum retinol concentration versus dark adaptation score. 1, Treated Nepali pregnant woman (NNIPS) (65). 2, Untreated Nepali pregnant women (NNIPS) (65). 3, Treated XN/XB Indonesian children (65). 4, Untreated XN Nepali pregnant women (L. Pizzarello, unpublished observations). 5, Untreated XN Nepali pregnant women (P. Christian, unpublished observations). 6, Untreated XN/XB Kenyan children (L. Nelmann, unpublished observations). 7, Untreated XN/XB Indian children (64). XB, Bitot spots; XN, night blindness.

placebo in the NNIPS-2 trial. These women had the best score among any population we have tested for whom clear evidence of the harmful effects of vitamin A deficiency was present, in this case significantly elevated maternal mortality rates (45). The clearly normal cutoff is based on the mean for pregnant Nepali women receiving weekly dosing (7000 RE of retinyl palmitate) with vitamin A in NNIPS-2. The rationale for this cutoff is that this score is achievable by a population known to be at risk for vitamin A deficiency in the absence of adequate intervention, whose rate of XN was significantly reduced and whose vitamin A status improved (but was not normalized) by such an intervention. The population mean has been used in setting this cutoff instead of the proportion falling below a particular value, as is done with measurements of serum retinol, because the proportion of such individuals might be small in borderline populations, which potentially could inflate the sample size requirements for testing.

When these cutoffs are applied to the 11 populations for which data are presented in Figure 1, 3 of 3 known normal populations are in fact identified as normal, and 8 of 8 known deficient populations are correctly identified as deficient.



**FIGURE 3** Compliance with pupillary versus conventional dark adaptation testing by age. Difference between the two curves is statistically significant ( $P < 0.0001$ , chi-square)

### Limitations of pupillary dark adaptation

Our work in India (64) and that by M. Haskell in Nepal (unpublished observations) suggest that the time course for recovery of the normal pupillary response after dosing of deficient individuals with vitamin A may be as long as 4–6 wk. Thus, programs wishing to use pupillary dark adaptation as an outcome indicator in program assessment likely need to wait at least this long before retesting treated individuals.

The time required to test a subject with this technique, including explanation, bleaching, dark adaptation and testing, is ~20 min. Thus, rapid testing of large numbers of persons is not practical. However, sample size calculations reported by us (64) suggest that in very deficient populations no more than half a dozen subjects would need to be tested to demonstrate that the group mean differed significantly from normal, although considerably larger numbers would be needed to ensure a representative sample. In even mildly deficient populations, testing of fewer than 100 subjects would be sufficient.

The test also requires preparation of an area that has been rendered sufficiently dark. We (64) have described the fabrication of a portable tent for use with the technique and have also provided details of a simple test that may be carried out to ensure adequate darkness of a testing area (56,64). But this can be cumbersome when moving across rural, hot, tropical areas.

This technique requires training (1–3 d) in the recognition of a pupillary response. We have trained social workers, non-ophthalmic nurses and a wide variety of other persons with no experience in the observation of the eye. Our experience suggests that most observers can make highly reliable measurements with this technique after only a brief period of training.

### Implications of pupillary dark adaptation screening for vitamin A programs

As a tool for population assessment, pupillary dark adaptation offers several advantages over other techniques: it is rapid, noninvasive, inexpensive and highly acceptable to target populations, and it does not require transport of samples. Furthermore, it appears able to detect subclinical vitamin A deficiency diseases: scores were significantly abnormal in an unintervened population of pregnant Nepali women in an area where clinical XN has been reported in only 11% of pregnant women (67). In contrast, arranging for a comfortable, darkened testing facility, although possible, can pose logistic challenges in hot, rural environments. Nighttime testing, where practical, might offer a practical alternative.

The technique lends itself to baseline population assessment and evaluation of the effect of intervention programs. To make maximum use of this technique, it is necessary to build a database of tested populations to develop a meaningful context for interpretation of future results. Less cumbersome means for achieving dark adaptation will make the test even more practical.

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