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D. W. Gregory
University of Aberdeen

L. Stankler
Aberdeen Royal Infirmary

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ZINC DEFICIENCY : SPECIFIC SCALP HAIR DEFECTS SEEN BY SCANNING ELECTRON MICROSCOPY MAY PROVIDE A VALUABLE NEW TEST

D.W.GREGORY[1*] and L.STANKLER[2]

Department of Bacteriology[1], University of Aberdeen and
Department of Dermatology[2], Aberdeen Royal Infirmary
Foresterhill, Aberdeen AB9 2ZD, Scotland

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Abstract

Zinc deficiency in man results in multisystem disease. It may be acquired or hereditary; the latter can be fatal if left untreated. Premature babies are particularly susceptible to zinc deficiency. Unfortunately no simple, reliable test for zinc status exists at present.

Short, newly-emerging scalp hair samples from 3 classical cases of zinc deficiency all showed the same characteristic abnormalities when examined by scanning electron microscopy, i.e. straight, blunt tips bearing scales, unusually thick cuticular scales with jagged free-edges, and very fine longitudinal corrugations in individual scales. These abnormal features occurring together appear to be specific for zinc deficiency; they also varied in severity with marked variations in zinc status during follow-up studies.

Due to the relative rarity of classic cases of zinc deficiency, it is not possible for one centre of our catchment size to conduct a pre-planned study. However, if the present findings can be confirmed elsewhere, it is concluded that scanning electron microscopy of appropriately-selected hairs may provide a valuable new test for the diagnosis of zinc deficiency and for monitoring the response to zinc therapy.

KEY WORDS: Zinc deficiency, Acrodermatitis enteropathica, Zinc status monitoring, Plasma zinc level, Zinc therapy, Scalp hair defects, Hair cuticle abnormalities, Premature babies, Failure to thrive, Scanning electron microscopy.

*Address for correspondence:
D.W.Gregory
Department of Bacteriology
University of Aberdeen
Foresterhill, Aberdeen AB9 2ZD
Scotland. Phone No.(0224) 681818 Ext.52448.

Introduction

Zinc is important for many biological functions; its deficiency in man results in multisystem disease [3].

Acquired zinc deficiency due to an increased requirement and/or decreased availability produces a clinical picture which includes characteristic lesions of skin and mucous membrane, alopecia, diarrhoea, failure to thrive and low levels of plasma zinc and alkaline phosphatase (a zinc metalloenzyme). Premature babies are particularly susceptible to this condition [8,14], especially if a low zinc level is further depressed by special circumstances [3,13,14]. Zinc deficiency may impair the development and function of important organs including the brain [16,20]. Early recognition and treatment with zinc supplements usually results in very rapid improvement of the overt characteristic symptoms [22].

In the rare hereditary disease acrodermatitis enteropathica, zinc deficiency due to an unknown defect in its absorption from the small intestine produces a similar clinical picture [2,15]. If left untreated the disease usually has an intermittent progressive downhill course with a fatal outcome.

It follows that a simple, reliable test for zinc status would be very valuable; unfortunately no such test exists at present. Definitive diagnosis therefore is made usually on clinical criteria and the response of symptoms to zinc therapy. A low plasma zinc level is the test most commonly used as an indicator of zinc deficiency, but the level may vary with other factors, and the validity can be questioned because most of the total body zinc is intracellular [1]. Attempts to estimate the zinc content of various tissues have not proved fully satisfactory [4,10]; the taste test [7] has been reported as unreliable [12] and is obviously not suitable for babies. Metabolic balance and ^{65}Zn isotope studies are more reliable but can only be carried out in specialised centres.

Although abnormalities in hair from zinc deficient patients have been observed using polarised light microscopy [9,21], this paper is the first report on the scanning electron microscopical appearance of scalp hair from such

patients.

This report is not a pre-planned study: it presents the findings from patients seen by Dermatologists in our hospitals over the last $3^1/_2$ years; follow-up studies have not always been possible. Due to the relative rarity of this condition, and hence the infrequency of its occurrence within a catchment area of our size, verification by one centre could take many years to achieve. However our findings may be of such significant help to those responsible for the care of zinc deficient patients, as they have been to clinicians here, we believe that our observations should be reported now to see if they can be confirmed elsewhere.

Method

Short, previously uncut scalp hairs were fixed to 25mm diam. stubs with very small drops of colloidal silver adhesive. After drying they were sputter-coated with 20nm platinum, and examined in a JEOL JSM-35CF scanning electron microscope at 10kV.

Materials

The 3 index cases had all been premature babies who were first referred to Dermatologists at between $2^1/_2$ and 4 months old; all were found to have multiple skin lesions which responded rapidly to oral zinc therapy, and plasma zinc levels below the normal range of 13-21μmol/l. Table 1 summarises relevant data on these 3 patients.

Table 1. Summary of relevant data on 3 index cases from patient records.

Index case number :-	1	2*	3
Sex :-	M	F	M
Gestation (weeks) :-	28	32	29
Birth weight (g) :-	1240	1860	1490
Age at presentation (months):-	4	2.5	4
Plasma at presentation Zinc (μmol/l):-	3.6	10	3
Alk. phosphatase:-	low	low	high
Oral Zinc treatment** (mg/kg/day):-	2	1.5	loading dose, then 1→2
Time for skin lesions to resolve (weeks):-	1.5	2	1
Plasma after treatment Zinc (μmol/l):-	19.7	variable- 1st normal 22.9	21
Time (months):-	1	4	1

* Since this paper was written, further tests on Case 2 have shown that she is likely to be a case of the rare condition acrodermatitis enteropathica (inherited zinc deficiency).

** Given as zinc sulphate solution prepared from 'Solvazinc' effervescent tablets (AB Astra, Sodertalje, Sweden).

Case 1 presented with characteristic skin lesions on the buttocks, face and neck, oral lesions, sparse scalp hair, and failure to thrive. Following treatment the baby was thriving after 1 month. Case 2* presented with characteristic skin lesions on the buttocks, cheeks, and outer aspects of forearms, paronychia involving all digits, sparse scalp hair and failure to thrive. The patient was thriving 3 weeks after treatment. Case 3 presented with "bullous impetigo" of the face and right hand, and severe napkin dermatitis.

Scalp hair samples were taken from all 3 index cases before treatment; additional samples were obtained from Cases 1 and 3 during and after treatment.

As controls for comparison with these cases we obtained samples from 10 healthy full-term normal delivery babies a few days after birth. The reasons for this choice are mentioned in the Discussion.

We also received samples from (Case 4) a 5 year old boy (brother of Case 2) who had previously experienced a rash on the elbows and knees and had a plasma zinc level of 8.2μmol/l, and from (Case 5) a 61 year old female with acquired zinc deficiency due to inadequate replacement therapy whilst on prolonged parenteral nutrition.

Observations

The appearance of control hairs from healthy full-term normal delivery babies is shown in Figs.1a,b and c. The tips were tapered, often curved, and lacked well-formed cuticular scales (Fig.1a). At 200μm behind the tips (Fig.1b) cuticular scales were present, but the distance between their neighbouring free-edges was greater than occurred more proximally (cf. Fig.1c); however by 450μm behind the tips the free-edge spacing was approaching that seen in mature hair. Figure 1c shows the proximal end of a 15mm long control hair which has the normal appearance of mature scalp hair.

The appearances of hairs from all 3 of the index cases prior to treatment were very similar and are illustrated by Figs.2a,b and c. The distal ends of hairs from the zinc deficient babies were blunt-ended, straight, and possessed thickened cuticular scales with jagged free-edges and characteristic fine longitudinal corrugations (e.g. Fig.2a). At 200μm behind the present tips their cuticular scales were still unusually thick with jagged free-edges and the same characteristic fine longitudinal corrugations (e.g. Fig.2b). The proximal ends of the zinc deficient babies' hairs usually showed all the same abnormal cuticular scale features (e.g. Fig.2c), but sometimes the fine longitudinal corrugations were less frequent or absent at this end.

Hairs from these zinc deficient babies frequently showed small, irregular pieces were flaking off the free-edges of cuticular scales, as shown in Fig.3. Occasionally transverse cracks through the entire hair were seen causing short (e.g. 35-50μm) pieces to break off the hair

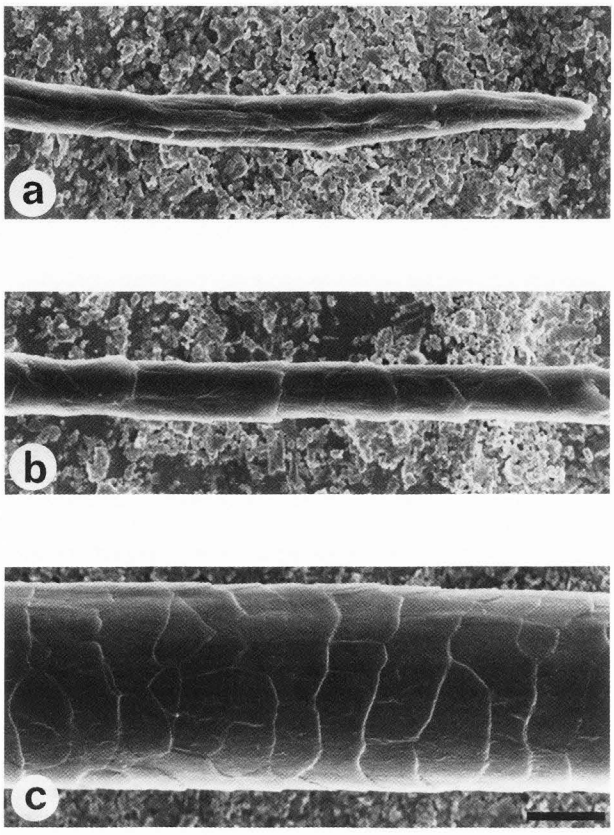

Fig.1. Typical appearance of control hairs from full-term normal delivery babies a few days after birth; (a) at the tip, (b) 200μm behind the tip, and (c) at the proximal end. Bar line (for all 3 figs.) = 10μm.

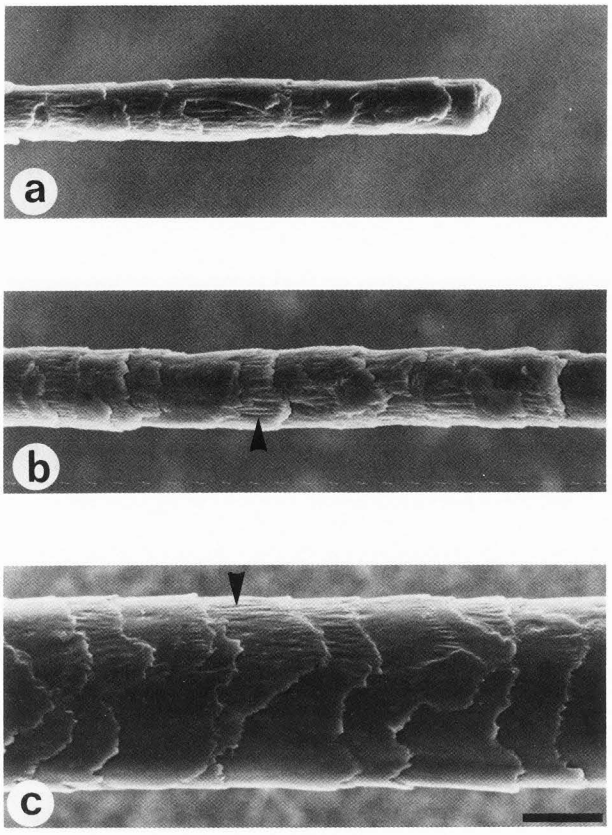

Fig.2. Typical appearance of hairs from the zinc deficient, index case babies at presentation; (a) at the present tip (Case 1), (b) 200μm behind the present tip (Case 1), and (c) at the proximal end (Case 3). Note the fine longitudinal corrugations (arrowed) on individual cuticular scales. Bar line (for all 3 figs.) = 10μm.

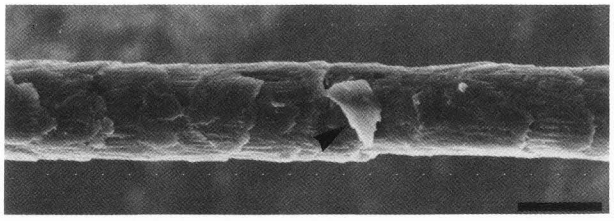

Fig.3. A small, spiky piece of cuticular scale (arrowed) about to flake off a hair from Case 1 at presentation (435μm behind the present tip) which shows how the jagged free-edges of cuticular scales on hairs from zinc deficient babies could have arisen. Bar line = 10μm.

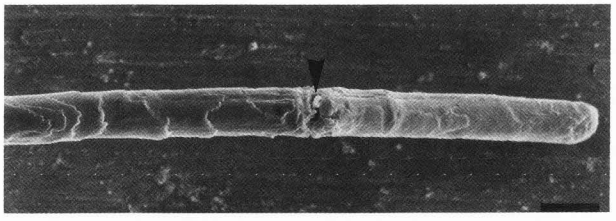

Fig.4. A hair from Case 1 with a major transverse crack (arrowed) which shows how distal fragments could break off hairs from zinc deficient babies giving rise to their truncated appearance with blunt tips. Bar line = 10μm.

tips, as shown in Fig.4 (see Discussion).

The progress of 2 of the index babies (Cases 1 and 3) was able to be followed after initial presentation and treatment. Samples of short, newly growing hairs taken from Case 1 after 6 months zinc treatment, and from Case 3 one month after a $4^{1}/_{2}$ month period of zinc therapy (Figs.5a and b) showed a marked return towards normal (cf. Figs.5a and b with Figs.1a and b respectively); at these times their symptoms had

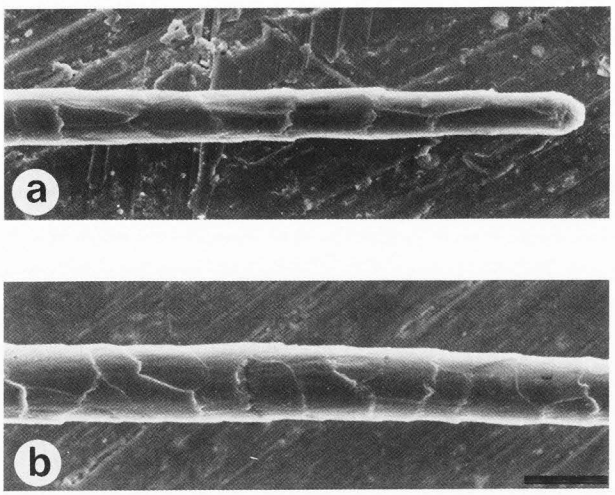

Fig.5. Typical appearance of hairs taken from Case 3 one month after a $4^1/_2$ month period of zinc therapy when the plasma zinc level had returned to normal (20μmol/l); (a) at the present tip, and (b) 200μm behind the present tip. Bar line (for both figs.) = 10μm.

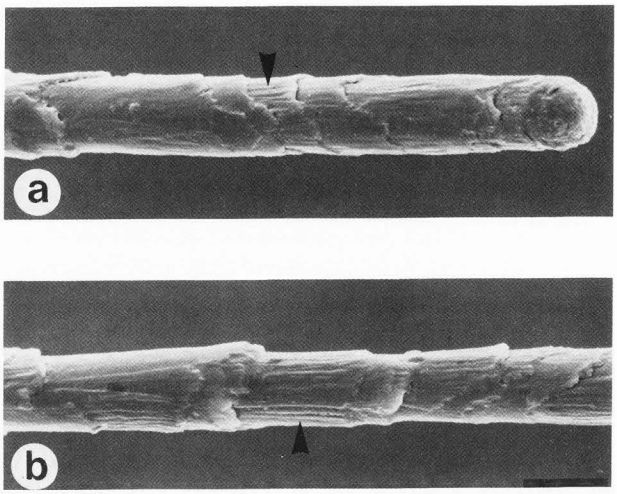

Fig.6. Typical appearance of hairs taken from Case 3 six months after zinc treatment had ceased when the plasma zinc level had reduced to 10μmol/l; (a) at the present tip, and (b) 200μm behind the present tip. Note the return of thickened cuticular scales with fine longitudinal corrugations (arrowed). Bar line (for both figs.) = 10μm.

resolved and their plasma zinc levels had returned to normal values (19.7μmol/l for Case 1 and 20μmol/l for Case 3).

However, further samples of short, newly growing hairs taken from Case 1 13 months after zinc therapy had stopped, and from Case 3 six months after zinc treatment had ceased (Figs.6a and b) showed a regression, with return of the characteristic cuticular abnormalities associated with zinc deficiency (cf. Figs.6a and b with Figs.2a and b respectively); by this time the plasma zinc level of Case 3 had progressively reduced to 10μmol/l, accompanied by a return to low plasma alkaline phosphatase levels.

The same characteristic hair abnormalities seen in the index cases were also observed in samples taken from a 5 year old boy with low plasma zinc level (Case 4) and a 61 year old woman with acquired zinc deficiency (Case 5).

Attempts were made to have hair samples from the present 5 cases analysed for amino acid composition, but the results were so unreproducible as to be unreliable (see Discussion).

Discussion

The 3 index cases described in this paper were classical examples of zinc deficiency, presenting with characteristic symptoms which quickly responded to zinc therapy, and low plasma zinc and alkaline phosphatase levels.

This paper describes for the first time a triplet of scalp hair features occurring together, i.e. straight, blunt tips bearing scales, unusually thick cuticular scales with jagged free-edges, and very fine longitudinal corrugations in individual scales - which were found in all 3 of these index cases. These abnormalities resolved as the zinc status of 2 patients improved, but returned when their zinc status worsened again. Furthermore the finding by scanning electron microscopy of similar abnormalities in the 5 cases described above of a rare condition also argues against a fortuitous association between these features and zinc deficiency. Thus it seems highly likely that the presence of these abnormalities can be used to diagnose zinc deficiency, and that their severity might be used to monitor zinc status.

This triplet of features occurring together has not been reported in any other condition. The fine longitudinal corrugations restricted to individual scales described in this paper are an order of magnitude finer than the coarse longitudinal ridging described in trichothiodystrophy [17] or the longitudinal flutings seen at the nodes in pili annulati [23 and personal observation (DWG)]; the latter are not only coarser but extend continuously over the length of many cuticular scales, and are probably due to visualisation through a damaged cuticle of the underlying cortical fibres. Similarly, the coarser longitudinal fissures seen in weathering [18] are due to visualisation of cortical fibres; although the jagged free-edges of cuticular scales seen in zinc deficiency are also a feature of weathering, the scales in this latter condition are not thickened as are those in the present report.

Conventional weathering is unlikely to have played a significant rôle in babies only a few months old. It seems that the jagged free-edges

of the abnormal cuticular scales described above are caused by small, irregular pieces of scale flaking off as shown in Fig.3. The reason why the remaining tips of the zinc deficient babies' hairs were blunt-ended and scale-bearing seems to be because short (e.g. 35-50μm) pieces of hair were breaking off at the tips due to the transverse cracking shown in Fig.4. Both these observations suggest these hairs were predisposed to minor trauma. Experimentally induced zinc deficiency in rats [11] produced a defective uptake of cystine, which is known to be a major constitutent of hair cuticle [5]. As reported above, attempts were made to have hair samples from the present 5 cases analysed for amino acid composition; the results were so unreproducible as to be unreliable, which is in keeping with the comments by other authorities [19] on the limited value of such analyses.

The fact that zinc deficient hairs may be occasionally shedding short fragments from the distal ends (Fig.4) does not invalidate the comparisons we have drawn between Fig.1b (200μm behind the original tip) and Figs.2b,5b and 6b (200μm behind the present tip). The actual position of comparison is not critical. It is obvious from the width of a tapering structure that these latter Figures are all taken within a few hundred μm of the original tip, whereas the features described extend for several mm and sometimes cm along the hair shaft. However it is our experience that the zinc deficiency features are most prominent near the hair tips. Because of the way hair grows the proximal end reflects the most recent metabolic status. Therefore, to maximise the chances of finding significant features which reflect recent zinc status, we strongly recommend that samples for examination should be obtained by selecting the most recently emerged, very short, previously-uncut hairs, severed as close as possible to the scalp, but studied most carefully towards their distal ends.

We purposely chose healthy full-term normal delivery babies as the controls for this study, despite the fact that the 3 index cases were all premature babies at birth. It is known that a high proportion of premature babies are in negative zinc balance [8], possibly because zinc is most rapidly taken up by the foetus during the last 2 months of pregnancy [14], so hair from premature babies would not have been a reliable control. The triplet of features reported cannot be ascribed to prematurity as they were also seen in a 5 year old boy (Case 4) and a 61 year old woman (Case 5). However our preliminary studies of hair taken from premature babies have revealed evidence of these zinc deficiency features in a significant proportion of cases, supporting the view that they are likely to be in negative zinc balance [8] and suggesting that at least mild zinc deficiency in premature babies may be more widespread than hitherto realised; this has important implications and is in keeping with the report that pregnant women may receive inadequate dietary zinc [6].

Conclusions

We have observed by scanning electron microscopy of scalp hair a triplet of abnormalities occurring together which seem to be associated with zinc deficiency; they were present in 5 cases of this rare condition and their severity varied with marked variations in zinc status. These features occurring together have not been reported for any other condition.

Hair is easy to obtain without upset to the patient, and can readily be transported, without deterioration, to a centre with scanning electron microscopy facilities. Scanning electron microscopy of scalp hair is a quick, simple and inexpensive procedure.

As the abnormal features were most marked near the hair tips, and because longer hair reflects an earlier metabolic status, it is important that samples obtained consist of the shortest, most recently emerged, uncut hairs which should be studied most carefully towards their distal ends.

If the present findings are confirmed this could provide a valuable new test for the diagnosis of zinc deficiency and for monitoring the response to zinc therapy. It may prove particularly appropriate for screening premature babies.

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References

1. Abdulla M. (1983). How adequate is plasma zinc as an indicator of zinc status? In: Zinc deficiency in human subjects, Prasad AS, Cavdar AO, Brewer GJ, Aggett PJ (eds), Alan R. Liss Inc, New York, 171-183.
2. Aggett PJ. (1983). Acrodermatitis enteropathica. J Inherited Metab Dis, 6 suppl 1: 39-43.
3. Aggett PJ, Harries JT. (1979). Current status of zinc in health and disease states. Arch Dis Child, 54, 909-917.
4. Anttila P, Simell O, Salmela S, Vuori E. (1984). Serum and hair zinc as predictors of clinical symptoms in acrodermatitis enteropathica. J Inherited Metab Dis, 7, 46-48.
5. Brown AC. (1971). Congenital hair defects. In: Birth defects, Original article series, 3rd Conference of the Clinical Definition of Birth Defects, Part XXI, 7, 52-90.
6. Bryce-Smith D, Simpson RID. (1984). Case of Anorexia Nervosa responding to zinc sulphate. Lancet, ii, 350.

7. Bryce-Smith D, Simpson RID. (1984). Anorexia, depression and zinc deficiency. Lancet, ii, 1162.
8. Cavell PA, Widdowson EM. (1964). Intakes and excretions of iron, copper and zinc in the neonatal period. Arch Dis Child, 39, 496-501.
9. Dupré A, Bonafé JL, Carriere JP. (1979). The hair in acrodermatitis enteropathica - a disease indicator? Acta Derm Venereol (Stockh), 59, 177-178.
10. Fell GS (1984). Diagnosis of zinc deficiency. In: Zinc in human medicine: Proceedings of a Symposium on the Role of Zinc in Health and Disease, Hambidge KM, Aggett PJ (eds), TIL Publications Limited, Isleworth and Toronto, 39-50.
11. Hsu JM, Anthony WL. (1971). Impairment of cystine-^{35}S incorporation into skin protein by zinc deficient rats. J Nutr, 101, 445-452.
12. Innes C. (1985). Affective illness and zinc deficiency. Lancet, i, 645-646.
13. Kay RG, Tasman-Jones C, Pybus J, Whiting R, Black H. (1976). A syndrome of acute zinc deficiency during total parenteral alimentation in man. Ann Surg, 183, 331-340.
14. Michie DD, Wirth FH. (1978). Plasma zinc levels in premature infants receiving parenteral nutrition. J Pediatr, 92, 798-800.
15. Moynahan EJ. (1974). Acrodermatitis enteropathica: a lethal inherited human zinc-deficiency disorder. Lancet, ii, 399-400.
16. Ohlsson A. (1981). Acrodermatitis enteropathica: reversibility of cerebral atrophy with zinc therapy. Acta Paediatr Scand, 70, 269-273.
17. Price VH, Odom RB, Ward WH, Jones FT. (1980). Trichothiodystrophy: Sulfur-deficient brittle hair as a marker for a neuroectodermal symptom complex. Arch Dermatol, 116, 1375-1384.
18. Rook A, Dawber R. (1982). Diseases of the hair and scalp. Blackwell Scientific Publications, Oxford, 227-232.
19. Rook A, Dawber R. (1982). Diseases of the hair and scalp. Blackwell Scientific Publications, Oxford, 35-36.
20. Sandstead HH, Gillespie DD, Brady RN. (1972). Zinc deficiency: effect on brain of the suckling rat. Pediatr Res, 6, 119-125.
21. Traupe H, Happle R, Gröbe H, Bertram HP. (1986). Polarization microscopy of hair in acrodermatitis enteropathica. Pediatr Dermatol, 3, 300-303.
22. Weismann K. (1980). Zinc metabolism and the skin. In: Recent advances in dermatology, Rook A, Savin J (eds), Churchill Livingstone, Edinburgh, 109-129.
23. Whiting DA. (1987). Structural abnormalities of the hair shaft. J Am Acad Dermatol, 16, 1-25.

Discussion with Reviewers

B.Forslind: Were the hair fibres plucked, and if so have the authors any comments concerning the appearance of the hair roots and the adhering inner root sheath?
Authors: The hairs were not plucked. They were cut as close as possible to the scalp, as we recommend in the Discussion.
M.Lindberg: You state in the discussion that the actual position of the site on the hair for comparison of abnormalities is not critical. Do you know if there are any changes in the hair dimensions in zinc deficiency and, if there are, has this any implication for the findings in your work?
Authors: We did not find any evidence for differences in width relative to distance from the tip (i.e. angle of taper) between zinc deficient babies' and normal hair. However, it is known that the rate of hair growth is depressed during zinc deficiency [4].
M.Lindberg: When you looked at your controls did you observe any differences in the hair morphology of the breast-fed children compared to those not breast-fed?
Authors: We did not make this comparison.
J.A.Swift: The authors have observed characteristic defects in the surface structure of scalp hairs from infants suffering from zinc deficiency. Whilst the SEM observations provide a useful indicator for zinc deficiency, is it not the case that some of the defects you describe might arise for reasons other than zinc deficiency (c.f. work of R.Dawber) and that further confirmation is required in all cases by direct analytical measurement of blood plasma or hair zinc levels? Since in all cases you saw hair surface defects, you also found low plasma zinc levels, is not the latter method of analysis the more reliable?
Authors: We have quoted plasma zinc levels where known and relevant. However, plasma zinc levels may not accurately reflect the more important tissue zinc levels [1,10], so they cannot be considered confirmatory, contradictory or reliable. Hair zinc levels are affected by hair growth rate [10], which in turn is depressed by zinc deficiency [4]. Rook and Dawber's comprehensive book [18 & 19] mentions some of the defects we describe occurring separately in other conditions, but, as stated in our Discussion, we are not aware of the triplet of features occurring together in any condition other than zinc deficiency.
J.A.Swift: Perhaps you can comment on the relative speeds and costs of the two methods of analysis (i.e. SEM versus chemical analysis).
Authors: Both tests could take anything from a few hours to a few days depending upon urgency relative to other staff and apparatus commitments. Given well-found laboratories with those types of test being carried out routinely, the costs of analysing one more sample is small in both cases; to provide an actual 'cost' would require very clear definition of which factors (such as consumables, staff salaries, equipment depreciation and maintenance etc.) were to be included.
J.A.Swift: You describe for one infant and the surface characteristics of the hair, initial defects, an improvement with zinc therapy and remission on withdrawal of zinc therapy. These observations were made on 'newly growing' hairs. Are you able to report seeing these transitions within the length of a single hair?
Authors: We report these progressive changes

for two infants. We do not report seeing these transitions conclusively within the length of a single hair. We sometimes observed apparent return towards normality moving proximally along single hair shafts taken from patients after treatment, which could have reflected the patients' improvement in zinc status; however, because the fine longitudinal corrugations were sometimes less frequent at the proximal ends of recently-emerged hairs even during a phase of zinc deficiency (as stated in our Observations and Discussion), such observations are difficult to interpret. Until the detailed reasons for the reported defects in hair surface morphology are properly understood, there is no reason to suppose that alterations to a single hair follicle are reversible.

J.A.Swift: I note that you mount the hairs for SEM observation without any pretreatment. By this process I believe that there is the possibility of misinterpretation of hair surface observations due to exogenous contamination (dirt, skin flakes, sebum, etc.). A procedure for overcoming such problems is to suspend the hair in a dilute solution of a surfactant and to sonicate with a low power ultrasonic cleaner (of the type often used for cleaning EM parts) for one minute, followed by repeated rinsing with distilled water over a period of one hour, then gently blot dry on paper tissue, allow to dry in air and then mount. In my experience the quality of SEM observations of human hair is much improved by such a cleaning process, whilst the sonication is sufficiently gentle as not to damage the hair surface itself.

Authors: We are confident that with high resolution electron microscopy we can distinguish between a clean hair surface and contaminating dirt, squamous epithelial cells, sebum, etc. - all of which we have seen and recognised. It is well known that cosmetic treatments, many of which contain surfactants, can cause weathering defects of hair [18], and we are not persuaded that ultrasonication could not modify the effects shown in Figs. 3 and 4.