reduction in the theoretical osmotic pressure gradient. The other solutes in the blood can also diffuse across the membrane, but their rates of penetration are slow relative to that of nitrous oxide (*9*). The true transient fluid shift is probably at least an order of magnitude less than the calculated maximum figure.

Thus, our prediction conflicts with the suggestion that gas-induced osmosis can cause transient changes in hematocrit value. To test this argument, we measured hematocrit values in eight surgical patients before and after the addition of 70 percent nitrous oxide to anesthetic mixtures of halothane and oxygen or isoflurane (Forane) (*10*) and oxygen (Table 1). In six other patients, anesthetized for a minimum of 2 hours with mixtures of isoflurane or halothane in 70 percent nitrous oxide, we measured the hematocrit value immediately before and after the elimination of nitrous oxide (Table 1). A nonrebreathing system was used in both experiments. There was a nearly instantaneous change in the concentration of the inspired gas (*11*). The corresponding change in alveolar gas required at least several breaths during which the partial pressure of nitrous oxide in the arterial blood only gradually increased. However, the time constant of this change was much smaller than the time constants of the processes for which gas-induced osmotic effects have been postulated (*1–5*). Hemoconcentration during nitrous oxide elimination or hemodilution during nitrous oxide inhalation would have supported the argument that gas-induced osmosis is important. We were unable to demonstrate any significant transient increase or decrease in hematocrit value for either experiment.

Since nitrous oxide appears to produce alpha sympathetic stimulation (*12*), shifts in the body fluid due to the cardiovascular effects of nitrous oxide may have masked those due to osmotic effects. However, the cardiovascular effects would be expected to occur more slowly than the transient osmotic effects. Conversely, a change in erythrocyte volume due to nitrous oxide would have magnified any change in hematocrit.

The in vivo results, together with our calculation of the maximum potential fluid shift, cast doubt on the importance of gas-induced osmosis during either hyperbaric exposure or clinical anesthesia.

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24 July 1972; revised 26 December 1972

Menkes' Kinky Hair Disease: Further Definition of the Defect in Copper Transport

Abstract. *Duodenal mucosa obtained from two patients with Menkes' syndrome contained abnormally large amounts of copper. The defect in copper absorption in this disease must lie in the process of intracellular handling or of transport across the serosal cell membrane. Fibroblastic cells cultured from the skin of patients and of heterozygous females show intense metachromasia in primary culture which disappears in subculture. These cells may be useful for the study of copper transport in vitro and for the identification of heterozygotes in affected families.*

Danks *et al.* (*1*) have demonstrated a defect in the intestinal absorption of copper in the X-linked inherited degenerative brain disease described by Menkes *et al.* (*2*). The arterial degeneration, cerebral degeneration, hypothermia, hair changes, and bone lesions seen in the disease can all be explained by copper deficiency (*3*).

We here report the results of further studies which show that the defect in copper absorption lies not in the uptake by the gut epithelium, but in the release of the copper from these cells, and suggest that a defect in copper transport may also exist in fibroblastic cells cultured from the skin of patients and of heterozygotes.

Samples of duodenal mucosa were obtained by an oral suction technique, with a Watson pediatric capsule being passed into the fourth part of the duodenum that had been treated with quinalbarbitone and metoclopramide. The mucosa samples weighed 2 to 3 mg (wet weight), and the copper content was determined by atomic absorption spectroscopy with the use of a carbon rod atomizer (*4*). The coefficient of variation in replicate samples was 6 percent. Samples were obtained from 17 babies and young children aged 10 months to 5 years during the investigation of mild persistent diarrhea. Only five of the biopsies showed any histological abnormality—celiac disease in two and giardiasis in four. The copper content of these samples ranged from 7.7 to 29.0 μg per gram (dry weight), the mean being 18.5 ± 6.3 (S.D.) μg per gram; the five that were microscopically abnormal were scattered throughout this range.

Three samples of duodenal mucosa, obtained from patient J.T. (*5*) at 6 and 9 months, contained 50, 60, and 76 μg per gram (dry weight). A sample from patient M.M. aged 10 months (*6*) contained 58 μg per gram (dry weight). Oral copper therapy had been withheld for at least 4 weeks before the samples were taken, and the levels are clearly abnormally high.

Considering this finding along with the previously published data showing defective absorption of radioactive copper from the intestine (*1*), we conclude that the defect in absorption lies not in uptake by the cells (that is, not in the brush border), but in transport within the cells or across the membrane on the serosal aspect of the cells. Very little is known about the intestinal absorption of copper (*7*). There is evidence of an active transport system in the upper intestine of the rat and a copper-binding protein has been demonstrated in the mucosal cells of the duodenum of the chick (*8*). If a similar protein exists in man it could be affected by this mu-

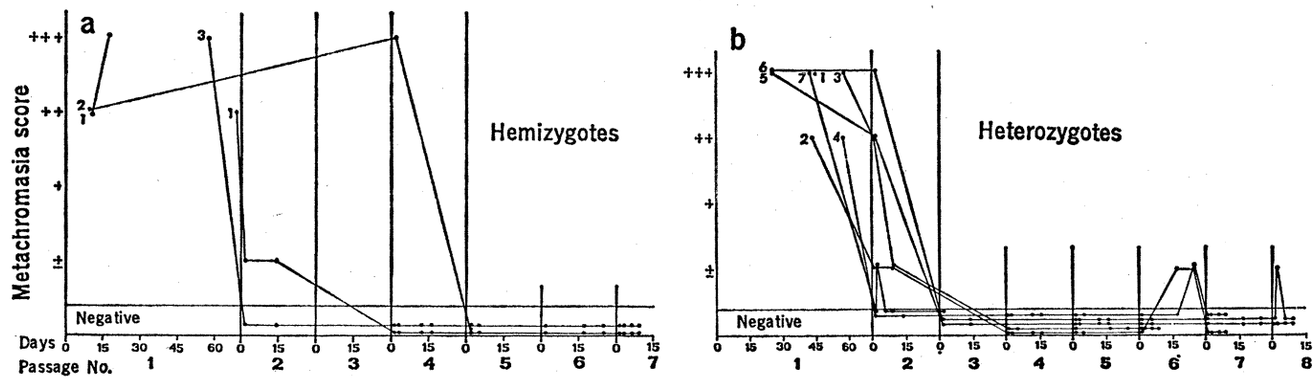

Fig. 1. Metachromasia scores during serial passages of fibroblastic cell cultures. (a) Cultures established from patients (hemizygotes): 1, J.T.; 2, P.D.; 3, M.M. (b) Cultures established from possible heterozygotes [1 and 2 (*12*)]; from obligate heterozygotes [3 to 7 (*13*)]. The dots depict the times when cover slips were stained and the results were observed; the lines are drawn merely to link results on each patient; ±, metachromatic granules present, but seen in less than 10 percent of cells; +, metachromatic granules present in 10 to 30 percent of cells; ++, metachromatic granules present in 10 to 30 percent of cells, and cells also vacuolated; +++, metachromatic granules present in more than 30 percent of cells.

tation. An alternative possible site for the defect is in the active transport process which presumably exists at the membrane on the serosal aspect of the cell.

It has been suggested that copper–amino acid complexes may be important for transport across cell membranes, especially copper-histidine complexes (*9*), and the important role of albumin as a carrier of copper in the serum is long established. An alteration in the formation of copper–amino acid complexes, or in the structure of albumin, could interfere with the transport of copper, and might explain the functional defect in these patients. However, we regard the latter explanation as unlikely in that the structure of albumin has been shown to be controlled by an autosomal locus (*10*) and this disease is caused by an X chromosomal mutation.

Skin samples were obtained from the volar surface of the forearm. They were rinsed in phosphate-buffered saline, diced finely, and stuck to cover slips with chick embryo extract and chick plasma clots in petri dishes containing Eagle's basal medium (BME) and 10 percent fetal calf serum. The copper content of the BME used was 1.3 μg per 100 ml. Three batches of fetal calf serum contained 304, 485, and 53 μg per 100 ml of copper. Unfortunately, three other batches used were not tested.

At the intervals indicated in Fig. 1, cover slips were rinsed briefly in saline and fixed in methanol for 5 minutes before staining in 0.05 percent toluidine blue in 0.02*M* benzoate buffer, *p*H 4.4. Metachromasia was scored semiquantitatively as follows: ±, metachromatic granules seen, but present in less than 10 percent of cells; +, metachromatic granules present in 10 to 30 percent of cells; ++, metachromatic granules present in 10 to 30 percent of cells, and cells also vacuolated, suggesting storage of excess membrane-bound material at some previous stage; +++, more than 30 percent of cells contain metachromatic granules. Seven cultures which showed metachromasia were stained with 0.05 percent Alcian blue in 0.025*M* sodium acetate–HCl buffer, at *p*H 5.7 in the presence of 0.1*M* and 0.3*M* magnesium chloride (*11*); but the granules did not stain.

Skin samples were cultured from three patients, J.T., P.D. (*12*), and M.M., from five obligate heterozygotes (*13*), and from two potential heterozygotes (*14*).

Metachromasia was seen in the toluidine blue stains of the cultured fibroblastic cells from all ten skin samples and followed a constant and very distinctive pattern (Fig. 1, a and b). All cultures showed intense metachromasia in more than 30 percent of cells in cover slips examined in the primary culture. This metachromasia diminished during the second passage in all except the culture from patient P.D., from which only three cover slips were stained. This pattern has not been encountered among cultures from 13 patients with various mucopolysaccharidoses, 7 with other heritable widespread disorders of connective tissue, and 50 normal individuals and other negative controls studied in our laboratory (*15*).

We therefore regard the presence of metachromatic granules in primary cultures, which clear during continued culture, as a distinctive phenotypic feature of cells carrying the Menkes' syndrome gene. This finding is of potential value in heterozygote recognition. More important, however, is the possibility that these cultured cells express the defect in copper transport and can be used in its elucidation. If this is true, an abnormality of membrane transport system for copper would seem the most likely defect in the intestinal cells.

We would hypothesize that the metachromasia is an indication of defective cellular metabolism consequent upon copper deficiency, and is gradually corrected by passive diffusion of copper from the medium during many days in culture. The copper content of the medium was generally high because of the large amount of copper in most batches of fetal calf serum. It is interesting that the three heterozygote cell lines which developed slight metachromasia again in late passages (Fig. 1) were all changed to media containing the batch with a low copper content (53 μg/100 ml) shortly before the metachromasia reappeared.

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15. E. Cartwright, unpublished.

4 October 1972; revised 27 December 1972 ■

Biological Activity of Insulin-Sepharose?

In a series of independent studies pertaining to the mechanism of action of insulin (*1*) and to altered sensitivity to insulin in both diabetes (*2*) and obesity (*3*), Cuatrecasas and co-workers reported the common premise in these studies to be that insulin elicits its biological actions by interacting with specific receptor sites located on the cell membrane. The key reference cited in each report (*1–3*) to justify this basic premise is a study by Cuatrecasas published in 1969 (*4*). In that study (*4*), evidence was presented which purportedly demonstrated retention of the hormonal activities of insulin covalently coupled to insoluble beaded agarose (Sepharose) polymers. On the basis of the relatively large size of the insulin-Sepharose bead as compared to the target intact fat cell, he concluded that the insulin molecule could not enter the intact cell and that therefore all of the metabolic effects of the hormone resulted exclusively from specific interactions with the cell membrane (*4*). The acceptance of this evidence as experimental confirmation of the unitary view of insulin action, in addition to the recent proliferation of studies on cell membrane insulin-binding sites attest to the importance that has been attached to these conclusions (*5*). However, calculations performed on the data taken from the original Cuatrecasas report (*4*), and developed in this comment, indicate a discrepancy that seriously questions the validity of the insulin-Sepharose results. Recent studies on insulin-Sepharose (*6, 7*) previously have cast some doubt on the interpretations of such experiments.

By calculating the amount of insulin coupled per bead of Sepharose, and comparing this with the amounts of immobilized insulin that were reported to be bioassayed (*4*), it appears that the total amounts recorded as assayed were, in many cases, less than the amounts of insulin coupled per bead. It therefore becomes questionable as to how this was operationally possible. As stated in table 1 of reference (*4*), the insulin-Phe-Sepharose and insulin-Lys-Sepharose (*8*) used in the study contained 320 and 360 μg, respectively, of crystalline zinc insulin coupled per milliliter of packed Sepharose. The lowest concentration reported for any other preparation was 171 μg/ml. We have been able to confirm that these concentrations of insulin bound to Sepharose indeed do result from the described conditions.

As was stated in that report (*4*), the Sepharose beads used ranged in particle size from 60 to 300 μm. This coincides with the range for Sepharose 2B in the swollen state (*9, 10*). Therefore, calculations of the maximum, minimum, and average number of beads theoretically capable of occupying a cubic centimeter can be made. We calculate (*11*) the maximum number that could theoretically be squashed (forced) into this space to be about 10^7 beads, based upon a spherical volume of $(4/3)\pi r^3$ per bead and a minimum diameter of 30 μm per bead. The latter value represents half of the minimum size for any of the available Sepharose preparations, according to determinations of the size distributions by the Pharmacia Company (*10*). The minimum number of beads per cubic centimeter would be about 4×10^4, assuming a maximum average size of 300 μm per bead and natural (unforced) sedimentation. On the basis of a "harmonic average" size of 163 μm per bead (*10*), there would be less than 10^6 beads per cubic centimeter of packed Sepharose. According to experimental evidence documented by the Pharmacia Company (*10*), sample batches of settled Sepharose 2B contain about 3.24×10^5 particles per milliliter. Neither we (*12*), nor the Pharmacia Company (*10*), could find any significant effects of cyanogen bromide treatment or insulin coupling on the particle size range of the Sepharose beads.

Therefore, on the basis of an activity of 25 international units per milligram of native crystalline insulin (used to convert micrograms to microunits), there would be from 0.85 to 200 microunits of insulin bound per bead of insulin-Sepharose. These values are derived from an average of 340 μg of insulin reported by Cuatrecasas to be coupled per milliliter of Sepharose for the insulin-Phe-Sepharose and insulin-Lys-Sepharose preparations. Thus, when the most reasonable number (about 5×10^5) of beads is assumed to be present per milliliter of Sepharose (which is derived from the harmonic average of the particle size distribution), there must be more than 10 microunits of insulin bound per bead.

According to Cuatrecasas (*4*), the insulin-Phe-Sepharose and insulin-Lys-Sepharose preparations had potencies, on intact fat cells, virtually equivalent to that of free native insulin on the basis of conversion of [^{14}C]glucose to $^{14}CO_2$, fatty acids, and glyceride glycerol and of suppression of hormone stimulated lipolysis. Yet, in all of these bioassays, in vitro, the total amounts of insulin-Sepharose reported to be present in the final incubation media [tables 4 and 5 and figure 2 in reference (*4*)] ranged from 2 to 2000 microunits. Most of the assays were conducted on insulin-Sepharose reported in total amounts ranging from 2 to 60 microunits per final incubation volume. Thus, in order to provide these insulin-Sepharose concentrations, only a few beads, and in many cases only a fraction of a single bead, would have had to have been present in the incubation vessels. Assuming the extremely unlikely circumstances that there was only the calculated minimum amount (0.85 microunits) of insulin associated with an average bead, there still would have had to have been no more than a relatively small number of beads present. Since it is likely that these adipocyte assays utilize about 10^4 to 10^5 cells per incubation volume cell size [50 to 100 μm (*4*)], the significance of any metabolic effects of such small numbers of beads (in most cases less than ten) would be subject to serious question. In an attempt to repeat the experiments of Cuatrecasas, one is faced with the