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# Evidence for Transfer of Enzyme Product as the Basis of Metabolic Cooperation between Tissue Culture Fibroblasts of Lesch-Nyhan Disease and Normal Cells

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**Abstract.** Tissue culture fibroblasts derived from patients with Lesch-Nyhan disease (congenital hyperuricosuria) have a reduced IMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.8) activity and therefore incorporate, as detected by radioautography, much smaller amounts of tritiated hypoxanthine or guanine into cell nuclei and cytoplasm than do normal cells. However, Lesch-Nyhan cells grown in close contact with normal fibroblasts incorporate these purines. This phenomenon, which requires cell to cell contact for correction of the mutant phenotype, has been called metabolic cooperation. After separation of Lesch-Nyhan cells from normal cells, there is a prompt reversion to the mutant phenotype although the transferase is stable under these conditions for many hours.

These results are most compatible with the transfer from normal to mutant fibroblasts of the product of the normal enzyme, a nucleotide or a nucleotide derivative, rather than the transfer of the transferase or informational macromolecules leading to the synthesis of the enzyme. Metabolic cooperation may provide a mechanism for maintaining normal cell function in the heterozygote *in vivo*. Evidence has been presented previously that selection of normal cells, presumably during embryogenesis, also provides a means for achieving normal function in the heterozygote.

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Congential hyperuricosuria (Lesch-Nyhan disease) is a sex-linked recessive disease resulting from a deficiency of IMP:pyrophosphate phosphoribosyltransferase activity (EC 2.4.2.8).[1,2] The basic mechanism responsible for the deficiency of the transferase activity (alternatively named hypoxanthine/guanine phosphoribosyltransferase, HGPRTase) is not known. The enzyme defect is demonstrable in cells grown in culture.[3–6] Normal cells ($HGPRTase^+$) incubated with tritiated hypoxanthine or guanine incorporate radioactive purines into intracellular nucleotides demonstrable by radioautographic methods. The enzyme defect in skin fibroblasts grown from patients with Lesch-Nyhan disease ($HGPRTase^-$) is readily identified by the marked reduction in radioactive nucleotides within these cells after similar treatment. Fibroblasts from heterozygotes reveal a mixed population as predicted by the Lyon hypothesis.[3,4,6]

A puzzling feature in the studies of heterozygotes has been the inability[7,8] of some laboratories to demonstrate clearly (by radioautography) a mixed population in fibroblast cultures unless cloning techniques were used. The reason for this was indicated in experiments with artificial mixtures of HGPRTase$^+$ and HGPRTase$^-$ cells.[4] As the cells proliferated and became confluent, fewer mutant cells were identifiable by this method. The same findings applied to the natural heterozygote, the mixed cell populations being most clearly demonstrated in sparse cultures. This observation suggested the occurrence of metabolic cooperation requiring cell-to-cell contact, originally described by Subak-Sharpe and his collaborators.[5,9,10]

The mechanism responsible for metabolic cooperation is unknown, but an observation concerning the nature of the cooperation was made in the course of an earlier study on mixed cell populations.[4] After the mixed cell population had been permitted to grow to confluence, the cells were passed at low cell density and it was found that there was now a reversion within 24 hr to a phenotypically mixed population.[4] In the present study, mixtures of Lesch-Nyhan cells and normal cells that had undergone metabolic cooperation were separated and incubated with labeled hypoxanthine for 2 hr. There was a prompt restoration of the mutant phenotype, even though HGPRTase is a rather stable enzyme under these conditions. These results are most compatible with the concept that the product of the transferase (or some derivative of the product) is transferred from normal to mutant cells by cell contact.

**Materials and Methods. Tissue culture:** HGPRTase$^-$ human fibroblast strains were established from patients with Lesch-Nyhan disease. Human HGPRTase$^+$ cell strains were derived from the skin of normal male infants as previously described.[4] Cells were grown in Waymouth's medium containing 10% fetal calf serum and antibiotics (penicillin 50 units, streptomycin 50 $\mu$g, and kanamycin 30 $\mu$g/ml). For each experiment, replicate cell populations of Lesch-Nyhan or normal fibroblasts and 1:1 mixtures of the two were grown on coverslips or in flat-bottomed glass bottles. Suspensions were prepared by detaching confluent cell monolayers from glass surfaces with 0.04% trypsin (Grand Island Biological Co.) and 0.02% disodium EDTA in Puck's saline A.[11] The cell suspension was diluted in an equal volume of complete medium and the cells were incubated in 5-ml siliconized Erlenmeyer flasks in a gyratory shaker in a water bath at 36°C for 2 hr. Examination of the cell suspension during and following incubation showed that over 90% of the cells remained single.

Coverslip cultures were grown on 6 × 30 mm coverslips placed in the flat-bottomed glass bottles used for growing cells for suspension cultures, or in 14.5 × 1.5 cm test tubes. Before confluent cell monolayers were harvested from glass bottles, the coverslips were removed and placed in individual test tubes. These coverslips were therefore representative of the cell monolayer prior to trypsinizing.

**Radioautographic studies:** Radioactive purines were chromatographed prior to use. [$^3$H]adenine (New England Nuclear Corp.) was sufficiently pure to use as received, but [$^3$H]hypoxanthine (New England Nuclear Corp.) required further purification by chromatography on Dowex 50.

Radioautographic studies were performed by adding [$^3$H]hypoxanthine (3 Ci/mmol) to a final concentration of 12.5 $\mu$Ci/ml for suspension cultures or 100 $\mu$Ci/ml for monolayers. [$^3$H]adenine (6 Ci/mmol) was added at a final concentration of 12.5 $\mu$Ci/ml to suspension cultures or 50 $\mu$Ci/ml to monolayer cultures. Sufficient [$^3$H]hypoxanthine was used to produce heavy labeling (30 grains or more per nucleus) in about 90% of HGPRTase$^+$ cells and less than 10% of HGPRTase$^-$ cells. Suspensions of cells incorporate isotope far more efficiently than monolayer cultures.

Cells grown on coverslips were incubated for 1.5 hr in medium containing the labeled purine, in an air incubator at 36°C. The coverslips were prepared for radioautography as previously described.[4]

Suspensions of cells were incubated with radioactive substrate for 2 hr. The cells were collected by centrifuging for 2.5 min at 700 rpm and washed twice with fresh medium without serum and once with Puck's saline. Siliconized tubes and pipets were used throughout. The cell pellets were fixed overnight with ethanol–acetic acid 3:1. Slides were prepared from the cell pellet and radioautographs were made as previously described.[4]

The radioactive grains in cell nuclei of 200 cells on each slide were counted. The cells were classified into three groups: <10, 10–30, and over 30 grains/nucleus. This classification indicates the ability of cells to convert hypoxanthine to inosinate or adenine to adenylic acid and is a measure of HGPRTase activity and adenine phosphoribosyltransferase (EC 2.4.2.7, APRTase) activity, respectively. Incubation of cells with [$^3$H]adenine provides a control of cell viability, metabolic activity, and availability of phosphoribosylpyrophosphate (PRPP), since APRTase carries out an analogous reaction to HGPRTase. We have found that APRTase activity in Lesch-Nyhan fibroblasts is not increased, differing in this respect from the mutant erythrocytes.

**Assay of HGPRTase and APRTase activity:** Enzyme assays were carried out on supernates of cells disrupted by freezing and thawing three times, followed by sonication for 30 sec at 9 kc/sec. Cell membranes and debris were removed by centrifuging at 25,000 × g for 3 min. The supernate was used for enzyme assays and protein was measured by the method of Lowry *et al.*[12] HGPRTase and APRTase activity were assayed by the method of Rubin *et al.*,[13] with either [8-$^{14}$C]hypoxanthine or [8-$^{14}$C]adenine as substrate.

**Results.** HGPRTase$^-$ cells in monolayer incorporate little hypoxanthine (Fig. 1*a*) when compared to HGPRTase$^+$ fibroblasts (Fig. 1*b*) and as shown quantitatively in Table 1. However, when HGPRTase$^-$ cells are mixed 1:1 with HGPRTase$^+$ cells and are grown to confluency, nearly all cells incorporate the labeled purine (Fig. 1*c* and Table 1). If confluent cultures of either HGPRTase$^+$ or HGPRTase$^-$ cells are trypsinized, suspended in culture medium, and incubated with [$^3$H]hypoxanthine, the characteristic incorporation of label into HGPRTase$^+$ cells (Fig. 1*e*) and the reduced incorporation into HGPRTase$^-$ mutant cells (Fig. 1*d*) is unchanged (Table 1). Similar experiments were done with mixed populations of HGPRTase$^+$ and HGPRTase$^-$ cells. When confluent, on a coverslip, most cells had incorporated isotopes. When these cells were trypsinized, suspended in culture medium, and incubated with hypoxanthine, approximately 50% of the cells were labeled (Fig. 1*f* and Table 1). Three other experiments gave similar results.

Control studies using [$^3$H]adenine with cells in monolayer and in suspension demonstrated good labeling of both HGPRTase$^+$ and HGPRTase$^-$ cells under both conditions of culture. Figs. 1*g*, 1*h*, and 1*i* show that suspension cultures actively incorporate this label and indicate that the change in hypoxanthine incorporation of mixed, normal, and mutant cell populations between monolayer and suspension culture does not reflect a change in viability or lack of availability of co-substrate (PRPP).

Enzyme stability in tissue culture was estimated by inhibiting protein synthesis with cycloheximide and determining the rate of fall of enzyme activity (Table 2). Under these conditions HGPRTase activity fell only slightly over a period of 12 hr, whereas APRTase proved unstable in fibroblasts, as shown by the marked

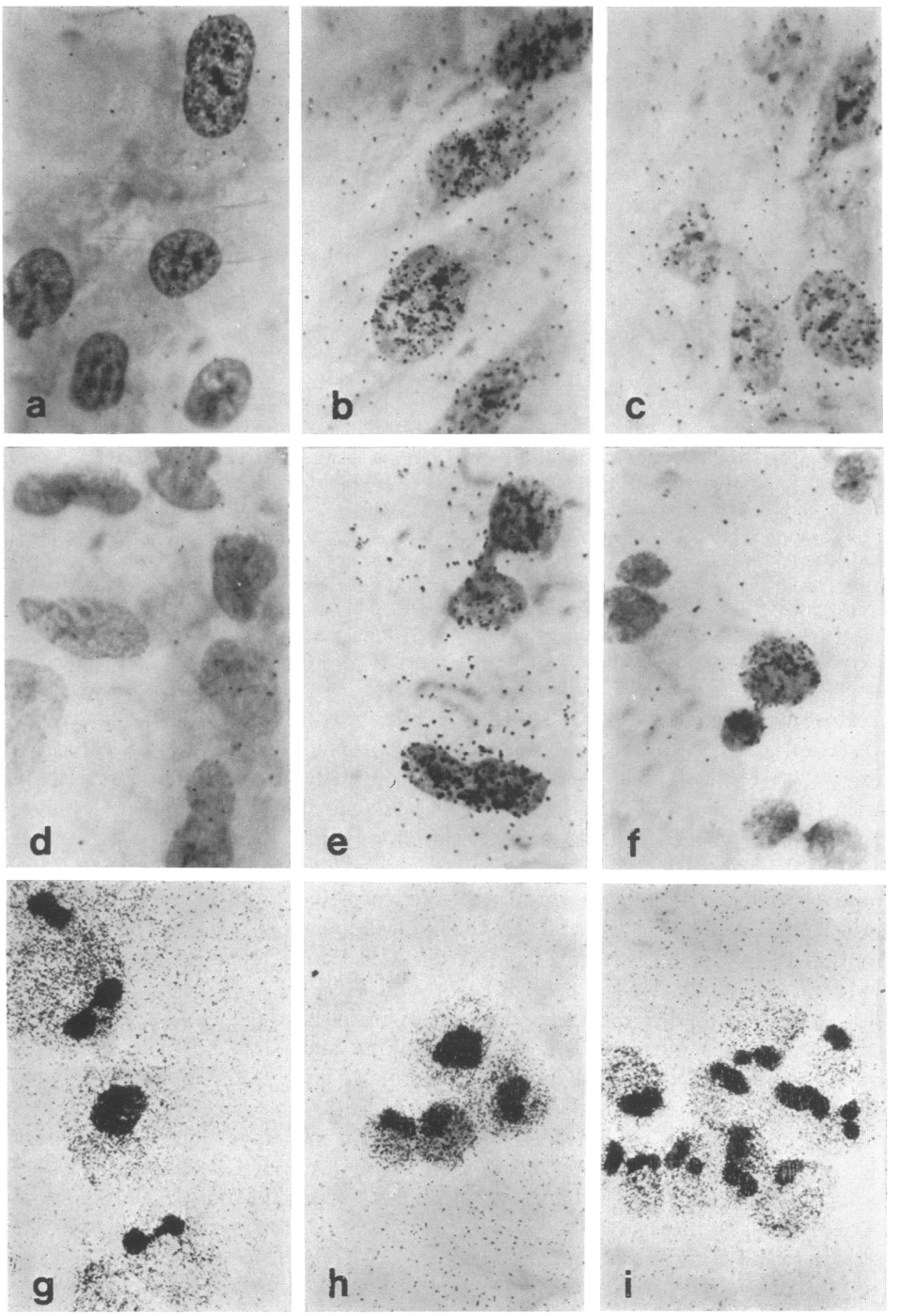


FIG. 1. Autoradiographs of human skin fibroblasts incubated with tritium-labeled hypoxanthine or adenine.

*Top row:* Monolayer cultures incubated with [$^3$H]hypoxanthine (×566). (*a*) Lesch-Nyhan, (*b*) Normal, (*c*) 50–50 mixture.

*Middle row:* Suspension cultures incubated with [$^3$H]hypoxanthine (×566). (*d*) Lesch-Nyhan, (*e*) Normal, (*f*) 50–50 mixture.

*Bottom row:* Suspension cultures incubated with [$^3$H]adenine (×278). (*g*) Lesch-Nyhan, (*h*) Normal, (*i*) 50–50 mixture.

TABLE 1. *Radioautographic analysis of [$^3H$]hypoxanthine incorporation into nuclei of human skin fibroblasts: normal (HGPRTase$^+$), mutant (HGPRTase$^-$), and 1 : 1 mixtures.**

| Human skin fibroblast strain | Grains/cell nucleus <10 | 10–30 | >30 |
|---|---|---|---|
| *Coverslip culture*† | | | |
| HGPRTase$^-$ | 110 | 74 | 16 |
| HGPRTase$^+$ | 0 | 21 | 178 |
| 1:1 mixture | 10 | 116 | 74 |
| Expected distribution if no cell interaction | 55 | 47 | 97 |
| *Trypsinized cell suspension*‡ | | | |
| HGPRTase$^-$ | 185 | 15 | 0 |
| HGPRTase$^+$ | 9 | 35 | 156 |
| 1:1 mixture | 110 | 32 | 58 |
| Expected distribution if no cell interaction | 97 | 25 | 78 |

In the confluent coverslip culture of mixed HGPRTase$^+$ and HGPRTase$^-$ cells, there were few unlabeled cells (<10 grains) which indicates metabolic cooperation. On suspension, there was prompt reversion to the expected number of unlabeled cells, as seen in the lower panel.

* Grains in 200 cells were counted in each preparation.

† Monolayer cultures grown on coverslips were incubated with 100 μCi/ml [$^3$H]hypoxanthine for 1.5 hr.

‡ Suspension cultures were incubated with 12.5 μCi of [$^3$H]hypoxanthine per ml for 2 hr.

reduction in the activity of this enzyme (Table 2). After it was found that HGPRTase is stable for at least 12 hr in the presence of cycloheximide (Table 2), cell suspensions derived from confluent cultures that had undergone metabolic cooperation were incubated with hypoxanthine in the presence of cycloheximide. This experiment showed that approximately half the cells promptly reverted to the mutant phenotype, a result similar to that observed in the absence of cycloheximide (Table 1).

**Discussion.** The molecular basis of metabolic cooperation requiring cell-to-cell contact is not known. It has been suggested[9] that HGPRTase$^+$ cells may provide mutant HGPRTase$^-$ cells with a substance(s) that corrects the defect and enables mutant cells to synthesize a functional HGPRTase. This substance might be episomal DNA, informational RNA (messenger RNA), or a regulatory molecule that stabilizes or activates a mutant enzyme. A second possibility is that HGPRTase$^+$ cells may provide HGPRTase$^-$ cells with preformed enzyme and thereby confer the capacity to metabolize guanine and hypoxanthine. A third possibility is that normal cells synthesize the radioactive nucleotide, which

TABLE 2. *Effects of cycloheximide on the stability of HGPRTase and APRTase in human fibroblast cultures.*

| Incubation time (hr) with cycloheximide* | Specific activity HGPRTase$^+$ | Specific activity APRTase† |
|---|---|---|
| 0 | 10.3 | 10.7 |
| 4 | 9.8 | ... |
| 8 | 12.4 | 6.8 |
| 12 | 9.6 | 0.6 |

* Cycloheximide, 1.0 μg/ml, was added to cultures at time zero. Two replicate cultures were harvested at each time. This concentration of cycloheximide inhibited incorporation of [1-$^{14}$C]-leucine by 88% in these cell cultures within 2 hr.

† Specific activity in nanomol of substrate reacted per min per mg protein. Values are the average of duplicates, which agreed within 20%. Cycloheximide was added to tissue cultures to inhibit the synthesis of new enzyme and thus permit the estimation of survival of HGPRTase and APRTase.

is transferred to the mutant cells as the nucleotide or as a product of the nucleotide. This last mechanism would predict that incorporation of radioactivity into mutant cells would cease promptly after separation of HGPRTase$^-$ cells from normal cells. If either of the first two mechanisms were operative, incorporation of radioactive label into dispersed mutant cells should continue as long as functional enzyme persisted.

In the experiments presented, metabolic cooperation was demonstrated in confluent cultures and disappeared promptly on dispersing the cells into a suspension. This observation suggests the transfer of a radioactive product of HGPRTase from normal to mutant cell as the most likely explanation for metabolic cooperation. However, if the HGPRTase were very unstable, this could also account for the rapid reversion to the mutant phenotype.

The addition of cycloheximide to culture medium inhibits the synthesis of new enzyme, permitting the determination of the survival time of enzyme that is already present. Under these conditions the HGPRTase was stable for 12 hr, in contrast to the marked reduction in APRTase activity as shown in Table 2. However, this approach to determining enzyme stability has been criticized because it is possible that cycloheximide may inhibit the synthesis of factors that normally degrade the enzyme.[14] To resolve this issue, we suspended confluent cultures of a mixed population of normal and mutant cells in medium containing cycloheximide and then incubated with [$^3$H]hypoxanthine. Under these conditions any enzyme already present in mutant cells should remain stable for at least 12 hr. However, prompt reversion of mutant cells occurred. The most logical conclusion is that metabolic cooperation between HGPRTase$^+$ and HGPRTase$^-$ cells involves transfer of a radioactive product of the normal enzyme. The composition of this radioactive product is not known, although it is probably the nucleotide inosinate, or some derivative of inosinate. Previous studies using normal erythrocytes and amniotic cells incubated with tritiated hypoxanthine for 45 min have identified the product in these cells as the nucleotide.[15,16] However, it is possible in the present studies that the radioactivity is transferred from normal to mutant fibroblasts after further conversion of the nucleotide either to oligonucleotides or macromolecules.

Metabolic cooperation between HGPRTase$^-$ and HGPRTase$^+$ fibroblasts appears to take place through cell-to-cell contact as suggested by Subak-Sharpe.[10] In our present studies, attempts were made to induce metabolic cooperation by incubating HGPRTase$^+$ cells and HGPRTase$^-$ cells on slides side by side or by transferring medium containing [$^3$H]hypoxanthine from HGPRTase$^+$ cultures to mutant cells. Neither approach was effective in increasing the labeling of HGPRTase$^-$ fibroblasts. Cells are generally impermeable to nucleotides in solution.[17] The rapid transfer of a product of HGPRTase from cell to cell observed here may involve a different type of process permitted by cell contact, and may involve nucleotides in the form of macromolecules as well as simple nucleotides.

The mutant phenotype of skin fibroblast cultures derived from patients with certain other inborn errors of metabolism (for example, the Hurler syndrome) is corrected by incubating these cells with secretions from normal fibroblasts.[18] The factor in these preparations is a heat-labile macromolecule, probably a pro-

tein, that is transferred through the medium from normal to mutant cells.[19] This type of metabolic cooperation does not require cell-to-cell contact and appears to be similar to bacterial satellitism.[20]

The specificity of the transfer of products from one cell to another remains to be determined. Bürk, Pitts, and Subak-Sharpe have shown[21] that metabolic cooperation requiring cell contact is a bidirectional process, since all cells of two mutant hamster cell lines, one HGPRTase$^-$ APRTase$^+$, and the other HGPRTase$^+$ APRTase$^-$, when grown in confluent cultures incorporate both hypoxanthine and adenine.

The potential importance of metabolic cooperation *in vivo* is not known. Frost, Weinstein, and Nyhan have recently shown[22] that the skin of heterozygotes for Lesch-Nyhan mutation incorporate [$^3$H]hypoxanthine into all cells *in situ*. It is possible that normal cells are selected for survival over the mutant cells embryologically, as has been demonstrated in lymphocytes of heterozygotes with Lesch-Nyhan disease.[23] Metabolic cooperation as seen *in vitro* may be another mechanism for maintaining normal function in mutant cells of heterozygotes.

Abbreviations: HGPRTase, hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8); APRTase, adenine phosphoribosyltransferase (EC 2.4.2.7).

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[1] IMP (inosine monophosphate):pyrophosphate phosphoribosyltransferase, EC 2.4.2.8, is the enzyme that converts hypoxanthine or guanine to the nucleotide using phosphoribosyl pyrophosphate (PRPP) as cosubstrate.

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