STUDIES ON

PURINE METABOLISM

IN CULTURED HUMAN FIBROBLASTS

Martin P. Uitendaal

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF. DR. A.J.H. VENDRIK
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
WOENSDAG 14 JUNI 1978 DES NAMIDDAGS TE 4 UUR

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geboren te VELSEN

1978
druk: Stichting Studentenpers Nijmegen

VOORWOORD

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Het onderzoek, waarvan dit proefschrift een weerspiegeling is, is verricht op het Anthropogenetisch Instituut van de Katholieke Universiteit te Nijmegen en tijdens werkbezoeken aan de Unité Cytogenetique Experimental van het Institut Pasteur te Parijs en is financieel gesteund door FUNGO, Stichting voor Medisch Wetenschappelijk Onderzoek.

CONTENTS

INTRODUCTION

1. *The purine salvage pathway*

Purine nucleotides play a vital role in the metabolism of cells. First of all they are the precursors for the nucleic acids DNA and RNA. Therefore they are indispensable for the conservation of genetic information and for the cellular organisation of protein synthesis in all organisms. In the second place, ATP, the trinucleotide of the purine adenine, is very important as a source for chemically bound energy. Thirdly, the cyclic nucleotides cAMP and cGMP act as cellular regulators.

For these reasons a disturbance of the balance between the different purine nucleotides will have a profound effect upon the metabolism and organisation of cells.

Purine nucleotides can be synthesised from 5'-phosphoribosyl-1-pyrophosphate (PRPP) by a sequence of ten reactions, the de novo pathway, which result in the formation of IMP. On the other hand, the purine nucleotides can also be produced by the salvage pathway enzymes, which reuse purine bases and nucleosides that result from the breakdown of nucleic acids (fig.1).

This purine salvage pathway comprises among others the enzymes hypoxanthine phosphoribosyl transferase (HPRT; EC 2.4.2.8), adenine phosphoribosyl transferase (APRT; EC 2.4.2.7), adenosine deaminase (ADA; EC 3.5.4.4) and purine nucleoside phosphorylase (NP; EC 2.4.2.1) (see fig.1). Deficiencies for these enzymes are often associated with severe clinical symptoms.

HPRT catalyses the conversion of hypoxanthine and guanine to their respective nucleotides IMP and GMP (fig.1). Deficiency for the enzyme can lead to two clinically very different syndromes: the Lesch-Nyhan syndrome (1,2) and X-linked gout (3,4,5). In general, patients with the Lesch-Nyhan syndrome show no demonstrable HPRT activity in their red blood cell lysates, but marked exceptions have been reported (6,7). Also, the fibroblasts from such patients have HPRT activity (8,9). In patients with X-linked gout due to an HPRT mutation the HPRT activity in hemolysate goes from undetectable (10) to normal (11).

APRT catalyses the reaction of PRPP with adenine to AMP (fig.1). The deficiency for APRT is inherited as an autosomal recessive trait (12,13). Heterozygosity for APRT deficiency leads to partial APRT deficiency, usually lower than 50% of normal, but is

not necessarily associated with clinical symptoms such as gout (12,13,14,15,16). Homozygosity for APRT deficiency has been reported for three individuals, two of whom form stones of 2,8-dihydroxyadenine as a result of xanthine oxidase action on the excess adenine in these patients (12,13). The other patient, a brother of one of the other two, only excreted 2,8-dihydroxyadenine and had no clinical symptoms except for hyperlaxity of the joints (13).

The enzyme ADA deaminates adenosine to form inosine (fig.1). Deficiency for ADA is shown to be associated with severe combined immune deficiency (17). ADA activity is also abberant in lymphocytes from patients with various types of leukemia (18,19,20,21, 22,23,24) and in blood cells from patients with other forms of cancerous disease (25, 26).

Another enzyme of the purine salvage pathway, NP, can both use the ADA reaction product inosine to form hypoxanthine (NP-Ino) and catalize the reverse reaction (NP-Hx) (see fig.1). Guanosine and guanine are also substrates for NP in mammalian cells. Deficiency for NP in erythrocytes (27,28,29,30) and in cultured fibroblasts (30,31 32) has been reported to be associated with T-cell immunity defects.

Purified NP from several mammalian sources has a certain activity towards adenine (NP-Ad). This NP-Ad activity is 1630 times lower than NP-Hx activity in the same preparation from human erythrocytes (33). In experiments with intact cells indirect evidence could be given for the reverse reaction (NP-Ado) in mammalian cells (34,35). Adenine formation from adenosine has, however, never been demonstrated in mammalian cell lysates. It is not known whether these reactions are catalyzed by the same enzyme that also causes the NP-Hx and NP-Ino reaction. Almost all species of mycoplasma, the smallest freeliving prokaryotic organisms and notorious for frequent and silent contamination of animal cell cultures (36), do exhibit both NP-Ad and NP-Ado activity (37). In fact, measurement of NP-Ado activity in the medium of cell cultures is used as a test for mycoplasma contamination (37). It is not known whether this phosphorylase enzyme shows activity towards hypoxanthine and inosine.

For further information about purine metabolism in man and clinical implications of deficiency for its enzymes the reader is referred to a recent review (38).

2. *Aim of this study*

Because of its implication in the several genetic diseases mentioned above, purine metabolism in man has been the object of study of the Department of Human Genetics of

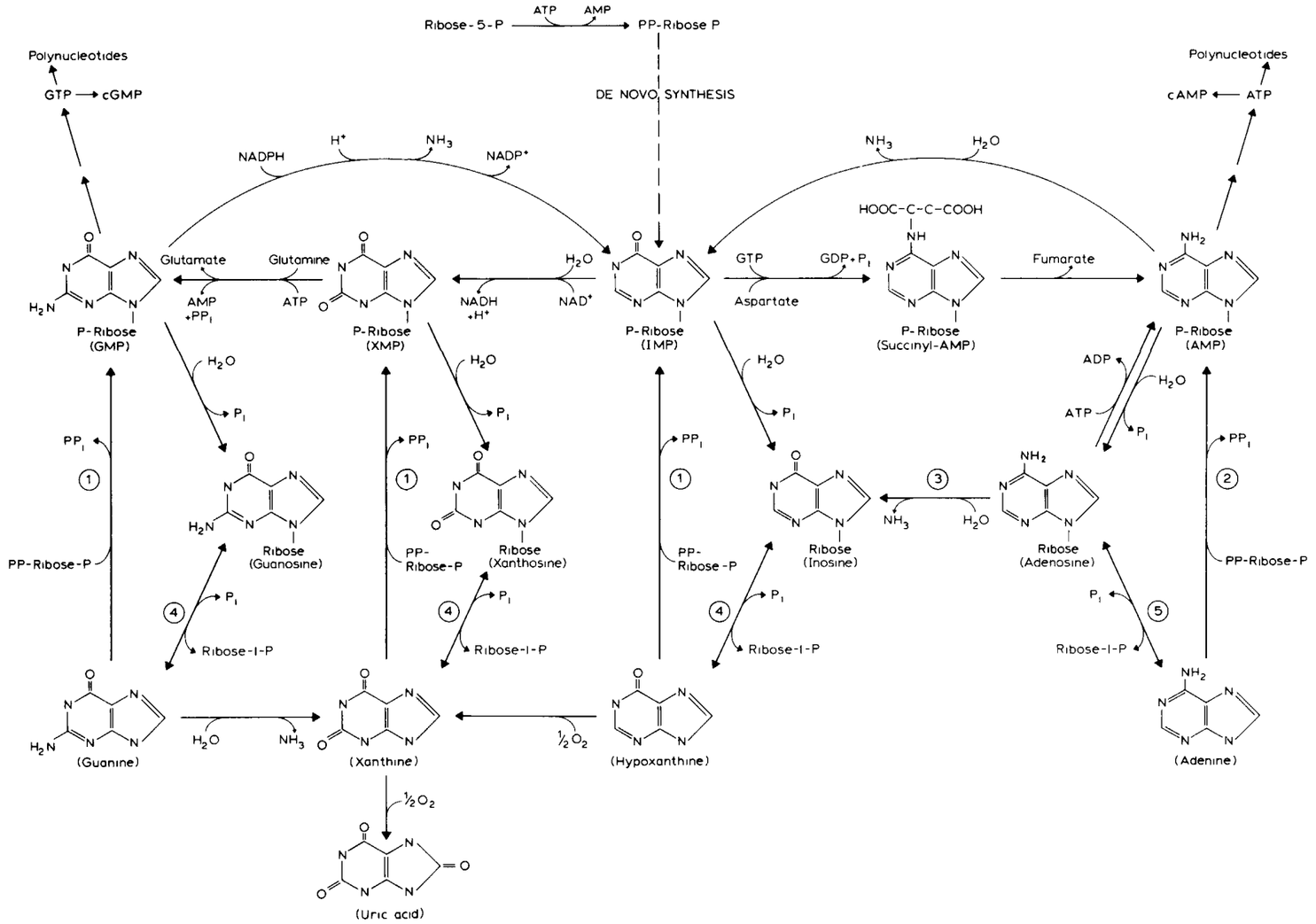

Fig. 1. Purine salvage pathway in human cells.

(1) Hypoxanthine phosphoribosyl transferase (HPRT)

(2) Adenine phosphoribosyl transferase (APRT).

(3) Adenosine deaminase (ADA).

(4) Purine nucleoside phosphorylase (NP).

(5) Adenosine phosphorylase (NP-Ad and NP-Ado).

the Nijmegen Medical Faculty since 1972 (39). The research project presented here, that was supported by a grant from FUNGO, Foundation for Medical Scientific Research in the Netherlands, was set up to further investigate purine metabolism in man, more specifically in cultured human fibroblasts. The application of ultramicromethods, that allow quantitative enzyme measurements in individual or small, counted numbers of cells (40, 41) had several advantages in these studies.

1. The blanks in the enzyme assays are mainly due to contamination of the radioactive substrates and consequently proportional to the amount of substrate added. Because the incubation volumes are extremely small (0.3 μl), a favorable signal:noise ratio can be obtained. Combined with long incubation times and high specific activities of the substrates this enables us to measure very low enzyme activities even in the 10^{-16} moles/hour range (paper 6).
2. Enzyme activities are related to the number of cells and not to the amount of protein, the latter parameter being unreliable because of its dependence on cell culture dynamics. In addition, the methodological error in the protein determination is avoided. These points are especially important in studies on gene expression, where small changes in enzyme activities have to be detectable (paper 5).
3. The procedure includes lyophilisation of the fibroblasts to be tested. This way of preparing cell lysates appears to be less damageing to enzyme activities than e.g. freezing and thawing under hypotonic conditions or sonication (42). This is especially important when mutant cells are tested with a less stable enzyme (paper 1,4).
4. Because assays can be performed in low numbers of cells, long culturing times to obtain large cell quantities can be avoided. This can be of help in prenatal diagnosis of inborn errors of metabolism, where only a limited amount of cell material is available and results must be obtained as sooh as possible (41,43). In genetic complementation analysis, single fused cells can be assayed directly without establishment of single vital clones (44).
5. Single, morphologically defined cells can be selected from a cell mixture. This permits the use of visually selected cell material which is essential in e.g. prenatal diagnosis of inborn errors of metabolism (41,43). Also the distribution of enzyme activity over the cells in different cell populations can be compared (paper 7).

Many enzymes can now be measured with both fluorogenic (41) and radioactive (44; papers 1,2,6,7) substrates on the ultramicro scale. Because of the points mentioned above many of the experiments in this study have been done using the ultramicromethod developed by Hösli (40,41).

The papers collected in this thesis can be divided into three categories:

a. Biochemical papers, describing techniques to measure ultramicrochemically several enzymes of the purine salvage pathway in cultured fibroblasts and reporting enzyme characterisation studies using these techniques. This cathegory comprises the papers 1,2 and 3.
b. Studies on gene expression. In these papers (4,5,6) alterations in cellular enzyme levels due to different causes are studied.
c. Papers about metabolic cooperation, dealing with different aspects of a phenomenon by which different cell types in contact influence each others metabolism (papers 7, 8,9 and 10).

3.1 Biochemical studies with the ultramicromethod

In paper 1 an ultramicro radiochemical assay for NP in cultured fibroblasts both with hypoxanthine and inosine as substrate, is described. In addition, The NP activities as measured with this method in fibroblasts from one of the first cases of NP deficiency, associated with T-cell immune defects, are given.
The ultramicrochemical ADA assay in cultured fibroblasts is given in paper 2. The main purpose of that paper is to describe a method to determine enzyme characteristics like pH optimum, Vmax, and Km using only 125 cells for each parameter. This method is illustrated with the partial characterisation of the purine salvage enzymes ADA and NP.

In paper 3 a more extensive ultramicro characterisation of human fibroblast NP is presented. The results of this study suggest that NP might play a regulatory role in the purine interconversion pathway in human fibroblasts.

Ultramicro characterisation of HPRT is described in paper 4.

3.2 Differences in expression of purine salvage enzymes

Paper 4 not only deals with the characterisation of HPRT in fibroblasts from normal individuals, but also compares the kinetics of normal HPRT with those of HPRT of a patient with X-linked gout. In addition, the heterogeneity in HPRT expression in different HPRT deficient patients and in different tissues is discussed on the basis of results of HPRT measurements in intact and lysed erythrocytes, lymphocytes and fibroblasts from one Lesch-Nyhan patient and three gouty patients.

Paper 5 reports a study on ADA activities in human fibroblasts. Although the ultra-

micromethod used appeared to be reproducible, statistically significant differences between ADA activities in different passages of the same cell strain were demonstrated. A possible explanation for these results on the basis of data, known from the literature, is discussed.

Mycoplasma contaminations in fibroblast cultures can imitate a reexpression of e.g. HPRT in Lesch-Nyhan cells (45). Since the studies reported here were done with cultured fibroblasts, the need was feld for a simple, sensitive test for mycoplasma contamination, if possible performed on the same culturing dish from which the cells to be studied were taken. Paper 6 describes such a test based on the assay for NP-Ad activity, that is much higher in mycoplasmas than in mammalian fibroblasts.

3.3 Metabolic cooperation between cells in culture

A special mechanism by which the expression of enzymes can be influenced is metabolic cooperation. This form of phenotypic interaction is defined as the process whereby the metabolism of cells in contact is modified by exchange of material (46,47). Paper 7 to 9 are concerned with this phenomenon as studied with HPRT deficient cells.

Metabolic cooperation has been studied most extensively in a cell system where both donor and recipient cells are cultured fibroblasts. It is believed to be caused by transfer of metabolites (48,49). In paper 7 the possibility of exchange of compounds, leading to enzyme activity, between fibroblasts is studied.

Paper 8 presents evidence for the hypothesis that metabolic cooperation can be caused by at least two mechanisms: transfer of metabolites and transfer of compounds leading to enzyme activity.

In paper 9 the occurence of these two forms of metabolic cooperation is investigated quantitatively in a system with normal erythrocytes as donor cells and HPRT deficient fibroblasts as recipient cells.

Paper 10 reviews the literature about metabolic cooperation in the light of the hypothesis of different forms of metabolic cooperation. The implication of differences in detection methods is discussed. A tentative classification of different forms of metabolic cooperation studied thus far is included.

Because of their involvement in a variety of genetical human disorders, the study of enzymes of the purine salvage pathway in cultured fibroblasts has a high clinical relevance. In addition, the purine salvage enzymes offer a very suitable model for e.g. gene expression studies and investigations on intercellular contact.

Biochemical studies with the ultramicromethod

Paper 1. A new ultramicrochemical assay for purine nucleoside phosphorylase.
Paper 2. Ultramicrochemical studies on enzyme kinetics.
Paper 3. Characterisation of purine nucleoside phosphorylase from fibroblasts using ultra-microchemical methods.

Differences in expression of purine salvage enzymes

Paper 4. Molecular and tissue specific heterogeneity in HPRT deficiency.
Paper 5. Fluctuating adenosine deaminase activities in cultured fibroblasts.
Paper 6. An ultra-microchemical test for mycoplasma contamination of cultured cells.

Metabolic cooperation between cells in culture

Paper 7. Metabolic cooperation studied by a quantitative enzyme assay of single cells.
Paper 8. Evidence for the existence of different types of metabolic cooperation.
Paper 9. Quantitative studies on two types of metabolic cooperation between normal erythrocytes and HPRT deficient fibroblasts.
Paper 10. Phenotypic interaction: Metabolic cooperation between cells in culture.

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A New Ultramicrochemical Assay for Purine Nucleoside Phosphorylase

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An ultramicrochemical method using radioactive substrates has been developed for the quantitation of purine nucleoside phosphorylase (NP) activity in single cells. NP activity can reproducibly be measured in both directions of the enzyme reaction. A quantitative relationship between the number of cells incubated and the NP activity was found. Fibroblasts from a patient with impaired T-lymphocyte function showed a substantial reduction of NP activity (both directions). In contrast to previously available methods, the present ultramicro approach makes it possible to analyze normal and immunodeficient single B and T lymphocytes.

Microchemical methods were successfully introduced into practical clinical work by Mattenheimer (1). Ultramicrochemical methods, measuring substances or enzyme activities in volumes lower than 10 μl, have been used in a limited way. As these methods are involved they have almost exclusively been employed in very special fields of experimental research (2). During the last decade a new and relatively simple approach to ultramicrochemistry was developed by one of us (3,4) and applied to diagnostic procedures, such as prenatal diagnosis and heterozygote detection (5,6), and fundamental investigations, such as genetic complementation analysis and regulation of gene expression (7,8). It is obvious that methods of this kind, enabling the measurement of enzyme activities even at the single cell level, provide new possibilities where conventional methods would not allow extensive analysis due to the limited amounts of material available.

Deficiency of an enzyme of the purine interconversion pathway, purine nucleoside phosphorylase (NP;EC 2.4.2.1), has been reported to be associ-

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ated with a disturbance of cellular immunity (9–11) The enzyme NP catalyzes the reversible reaction of the purine nucleosides inosine, xanthosine, and guanosine with inorganic phosphate to ribose 1-phosphate and hypoxanthine, xanthine, and guanine, respectively

In the present paper a simple ultramicromethod is described for the determination of NP activity in a small number [1–20] of visually selected fibroblasts using ^{14}C-labeled hypoxanthine (NP-Hx reaction) and ^{14}C-labeled inosine (NP Ino reaction) as substrates

METHODS AND MATERIALS

Incubation with lyophilized fibroblasts Fibroblasts were cultured in plastic film dishes (PFDs) as described previously (4) Before use the cultures were washed three times, shock-frozen in liquid nitrogen, and lyophilized overnight Subsequently, small plastic leaflets, carrying a counted number of visually selected cells, were cut from the plastic bottom of a PFD The leaflets were incubated in sealed Parafilm microcuvettes (PMCs 4) containing 0 3 μl of incubation mixture delivered with a gauged micro constriction pipet PMCs are small reaction vessels molded immediately before use in strips of conventional Parafilm

The reaction mixture for the NP reaction with hypoxanthine (NP-Hx) contained 0 17 M Tris–HCl buffer, pH 7 0, 0 5‰ (w/v) bovine serum albumin (BSA) to prevent surface denaturation of the enzyme, 0 8‰ penicillin, 0 8‰ streptomycin, 3 3 mM EDTA, to inhibit the hypoxanthine phosphoribosyl transferase reaction, 4 7 mM phosphate, and 0 13 mM [8-^{14}C]hypoxanthine (Radiochemical Centre, Amersham sp act, 50–52 mCi/mmol) For the reverse reaction with inosine as a substrate (NP-Ino), the incubation mixture contained 0 17 M Tris–HCl, pH 7 4, 0 5‰ BSA, 0 8‰ penicillin, 0 8‰ streptomycin, 3 3 mM EDTA, 1 7 mM ribose 1 phosphate, and 0 27 mM [8-^{14}C]inosine (Radiochemical Centre, Amersham, sp act, 50–52 mCi/mmol)

At the end of the incubation, the whole contents of the PMCs were placed on Whatman 3MM paper strips Substrates and products were separated by descending chromatography using 0 5 N ammonia and 0 05 N EDTA as a solvent (12) Unlabeled Hx and Ino were cochromatographed as reference compounds, and after visualization of purine spots under uv light, these spots were cut out and the radioactivity was quantified by means of liquid scintillation counting (Packard Model 2450 counter)

Calculation of enzyme activities The specific enzyme activity is

calculated from the net product formed and is expressed in 10^{-13} mol/cell/hr. The total input of radioactive substrate varies slightly for the individual incubations, this is due to micropipetting errors. There is some contamination (approximately 1%, varying from batch to batch) of substrate with product. To correct for this, the contamination in each individual incubation is calculated from the integral of product and substrate. This contamination (a certain percentage of the sum of product and substrate) is subtracted from the product. Therefore, the product measured in counts per minute is not directly representative of the product actually formed by the enzyme reaction (cf. Tables 1 and 2).

RESULTS

Of two control fibroblast strains, the NP-Hx and NP-Ino activities were determined in five replicates with five lyophilized fibroblasts per incubation mixture. With strain I the mean product measured in the NP-Hx reaction was 812 cpm (range, 620 to 969 cpm), corresponding to a

TABLE 1

REPRODUCIBILITY OF THE ULTRAMICROCHEMICAL PURINE NUCLEOSIDE PHOSPHORYLASE ASSAY IN FIBROBLASTS (HYPOXANTHINE AS A SUBSTRATE)[a]

Fibroblast strain	Assay No.	Product measured (cpm)	Enzyme activity ($\times 10^{-13}$ mol/cell/hr)
I	1	620	12.6
	2	854	19.9
	3	782	17.4
	4	969	19.0
	5	847	18.5
	Mean (± SD)		17.5 (± 2.9)
II	1	883	19.8
	2	870	17.6
	3	678	14.1
	4	757	16.3
	5	770	16.8
	Mean (± SD)		16.9 (± 2.1)

[a] NP-Hx activities in two control human fibroblast cultures. Five cells per assay were incubated for 30 min at 37°C. For calculation of enzyme activities see Methods and Materials.

TABLE 2

REPRODUCIBILITY OF THE ULTRAMICROCHEMICAL PURINE NUCLEOSIDE PHOSPHORYLASE ASSAY IN FIBROBLASTS (INOSINE AS A SUBSTRATE)[a]

Fibroblast strain	Assay No	Product measured (cpm)	Enzyme activity ($\times 10^{-13}$ mol/cell/hr)
I	1	411	4.5
	2	354	4.4
	3	364	3.5
	4	449	5 1
	5	385	4 5
	Mean (± SD)		4.4 (± 0.6)
II	1	400	5.3
	2	400	4 8
	3	395	4.7
	4	368	4.3
	5	503	6 7
	Mean (± SD)		5.2 (± 0.9)

[a] NP-Ino activities in two control human fibroblast cultures Five cells per assay were incubated for 45 min at 37°C. For calculation of enzyme activities, see Methods and Materials.

calculated enzyme activity of 17.5 $\times$ 10^{-13} mol/cell/hr (range, 12.6 to 19.9 $\times$ 10^{-13} mol/cell/hr). With strain II, the calculated enzyme activities

TABLE 3

RELATION BETWEEN THE NUMBERS OF CELLS INCUBATED AND PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY (INOSINE AS A SUBSTRATE)[a]

Number of cells tested	Incubation time (min)	NP-Ino activity ($\times 10^{-13}$ mol/hr)		
		Mean	Range	SD
1	120	5.9	2 8– 9.7	2.2 (37%)
2	120	10.5	7.5–14.4	2 9 (28%)
4	120	17.8	12.1–23 8	4 4 (25%)
8	60	37.6	30.0–50 9	9 0 (24%)
16	45	73.1	66.5–80.0	5.1 (7%)

[a] Effect of varying the number of cells incubated on the NP-Ino activity. For each number of cells, 10 independent isolations were done and the corresponding means, ranges, and standard deviations are given. Values in parentheses indicate the standard deviation as a percentage of the mean value.

ranged from 14.1 to 19.8 $\times 10^{-13}$ mol/cell/hr; the mean NP-Hx activity was 16.9 $\times 10^{-13}$ mol/cell/hr (Table 1).

The rate of the NP-Ino reaction was lower as compared to that of the NP-Hx reaction. For cell strain I, activities between 3.5 and 5.1 $\times 10^{-13}$ mol/cell/hr, with a mean value of 4.4 $\times 10^{-13}$ mol/cell/hr, were found. The activities of strain II ranged between 4.3 and 6.7 $\times 10^{-13}$ mol/cell/hr, the mean value being 5.2 $\times 10^{-13}$ mol/cell/hr (Table 2).

A quantitative relationship was observed between the number of cells incubated and the mean levels of activity of NP-Ino (Table 3). All these incubations were carried out in 10 replicates. The standard deviations as a percentage of the mean in incubations with small numbers of cells exceeded those in the assays with larger numbers of cells: With one cell the standard deviation was 37% of the mean value; with 16 cells 7% was found (Table 3). Similar results were obtained with the NP-Hx measurements (data not shown).

Fibroblasts from a patient with an immune disease, characterized by impaired T-lymphocyte function, were assayed in five replicates. The results show a deficiency for NP in both directions: For the NP-Hx reaction, an activity of 5.5 $\times 10^{-13}$ mol/cell/hr ($\pm$27% of the normal mean value) ranging from 4.0 to 7.9 $\times 10^{-13}$ mol/cell/hr was found; the NP-Ino activity was 1.5 $\times 10^{-13}$ mol/cell/hr ($\pm$26% of the normal mean value; Table 4), ranging from 0.75 to 1.65 $\times 10^{-13}$ mol/cell/hr.

TABLE 4

COMPARISON BETWEEN PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITY OF CONTROL FIBROBLASTS AND ACTIVITY OF FIBROBLASTS FROM A PATIENT WITH IMMUNE DISEASE[a]

	Enzyme activities ($\times 10^{-13}$ mol/cell/hr)	
	NP-Ino	NP-Hx
Control strains (n = 6)	5.5 (4.2–7.6)	21.1 (16.9–27.3)
Patient	1.5	5.5

[a] NP activities were determined in cultured control fibroblasts (six strains) and in fibroblasts from a patient with immune disease, characterized by a severely impaired T-lymphocyte function [Ref. (10)]. From each cell strain, five plastic leaflets, each carrying five fibroblasts, were tested, and the mean activities are given in 10^{-13} mol/cell/hr. Values in parentheses indicate the range of the mean activities found with the control strains.

DISCUSSION

The present radiochemical ultramicromethod makes it possible to assess NP activity quantitatively in small numbers of visually selected, lyophilized fibroblasts, even assays with a single cell can be carried out (Table 3) Preliminary experiments show, for example, that it is possible to study single normal and immunodeficient B and T lymphocytes with the present techniques Ultramicrochemical enzyme characterization can also be carried out With a total of a few hundred cells, K_m values and pH optima of fibroblast NP could be determined (12)

The method of Kalckar (13), frequently used for the determination of NP activity, is limited to the NP-Ino reaction It is based upon the spectrophotometric measurement of uric acid originating from hypoxanthine (the product of the NP-Ino reaction) in the presence of excess xanthine oxidase However, with the present method the activity of fibroblast NP with hypoxanthine as a substrate exceeds that with inosine as a substrate by a factor 3 to 5 (Tables 1–4) This has also been found with human erythrocytes (11), therefore, the NP-Hx reaction appears to be the most sensitive way to diagnose an NP deficiency and to eventually characterize residual NP activity

As is illustrated by the results in Table 3, the relative ranges of the enzyme activities in the measurements with one or two cells were considerably larger than those, for example, in the measurements with 8 or 16 cells This was to be expected, because the cells were isolated from asynchronously growing cultures The effect of cell-cycle-dependent fluctuations in enzyme activities is more pronounced when only a few cells are tested Similar observations have been made with other enzymes of purine metabolism (14) and with lysosomal hydrolases (4) For diagnostic purposes 10 replicate incubations are carried out with 5 or 10 cells to obtain a reliable mean value for the enzyme activity under study

The data summarized in Table 4 show that cells from normal controls and from an NP-deficient individual can clearly be distinguished A residual NP activity of approximately 27% of the normal mean value was observed in the patient's fibroblasts The deficiency for NP was seen in both directions of the enzyme reaction (Table 4), this finding is in agreement with the view that the conversion of hypoxanthine to inosine and the reverse reaction in human fibroblasts are catalyzed by the same enzyme (13) It should be noted that, using conventional assays, in lysates from erythrocytes and cultured fibroblasts of the NP-deficient patient hardly any NP activity could be detected, less than 2% of the

normal activity (10). These differences are due to the method used for the preparation of the enzyme sources: For the conventional assays, lysates were obtained by sonication of erythrocytes or trypsinized fibroblasts, whereas in the microassay growing cell cultures were lyophilized *in situ*. Experiments with normal and mutant cells have shown that the activities of several enzymes of purine metabolism were higher after lyophilization than after freezing or sonication (de Bruyn and Uitendaal, unpublished observations). Therefore, the activity of a mutant NP enzyme with decreased stability in the patient's cells might very well be less affected by lyophilization than by freezing or sonication.

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ULTRAMICROCHEMICAL STUDIES ON ENZYME KINETICS

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INTRODUCTION

Methods to measure enzyme activities in very small numbers of cells making use of radioactive substrate have been introduced for enzymes of the purine salvage pathway (1).

Two other enzymes of the salvage pathway, nucleoside phosphorylase (NP; EC 2.4.2.1) and adenosine deaminase (ADA; EC 3.5.4.4) have drawn our attention. Deficiency of ADA is shown to be associated with severe combined immunodeficiency (2) and ADA activity is also aberrant in various forms of leukemia (3,4). Recently, a deficiency of NP was reported to be associated with T-cell immunity defects (5).

In this communication a method to determine enzyme properties, such as K_M values and pH optimum, with only 125 cells is reported.

METHODS

The equipment and techniques for the ultramicrochemical measurements with the use of Plastic Film Dishes (PFD's) and Parafilm Micro Cuvettes (PMC's) have been described previously (6).

The reaction mixture for the NP reaction with hypoxanthine as a substrate (NP-Hx) contained 0.17 M Tris-HCl buffer (pH 7.0), 0.5 °/oo BSA, to prevent surface denaturation of the enzyme, 0.8 mg/ml penicillin, 0.8 mg/ml streptomycin, 3.3 mM EDTA to inhibit the

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hypoxanthine phosphoribosyl transferase reaction, 1.7 mM inorganic phosphate and 0.133 mM ^{14}C-labeled hypoxanthine. For the reverse NP reaction with inosine as substrate (NP-Ino) the reaction mixture was 0.17 M Tris-HCl buffer (pH 7.4), 0.5 °/oo BSA, 0.8 mg/ml penicillin, 0.8 mg/ml streptomycin, 3.3 mM EDTA, 1.7 mM ribose-1-phosphate and 0.267 mM ^{14}C-labeled inosine. Reactions were terminated by pushing out the total contents (0.3 µl) of the PMC's onto Whatman 3MM paper strips (1). Substrate and reaction products were separated by descending chromatography with a 0.5 N ammonia, 0.05 N EDTA solution as eluent.

The ADA reaction mixture contained 0.17 M Tris-HCl buffer (pH

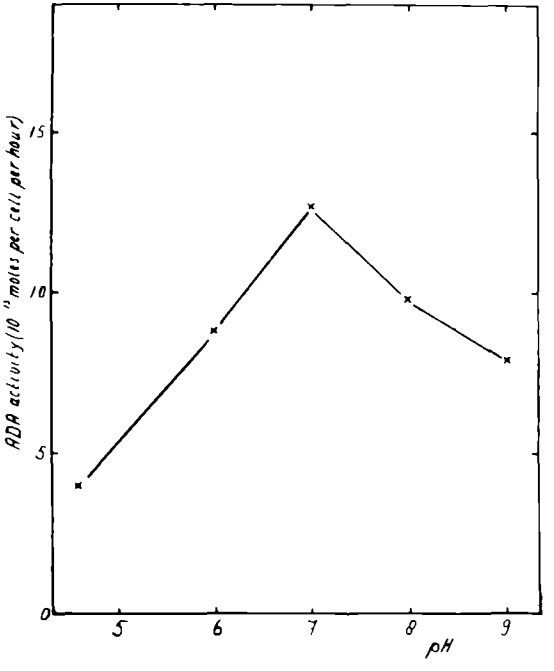

Figure 1.
pH dependence of ADA from normal human fibroblasts. Each point represents an average of five measurements.

7.4), 0.5 °/oo BSA, 0.8 mg/ml penicillin, 0.8 mg/ml streptomycin, 3.3 mM EDTA and 0.133 mM ^{14}C-labeled adenosine. Separation of substrate and product was performed on DEAE paper with 1mM ammonium formate as eluent.

Unlabeled reference compounds were cochromatographed to visualise after separation the spots under U.V. light. These spots were cut out and counted in a liquid scintillation counter (1).

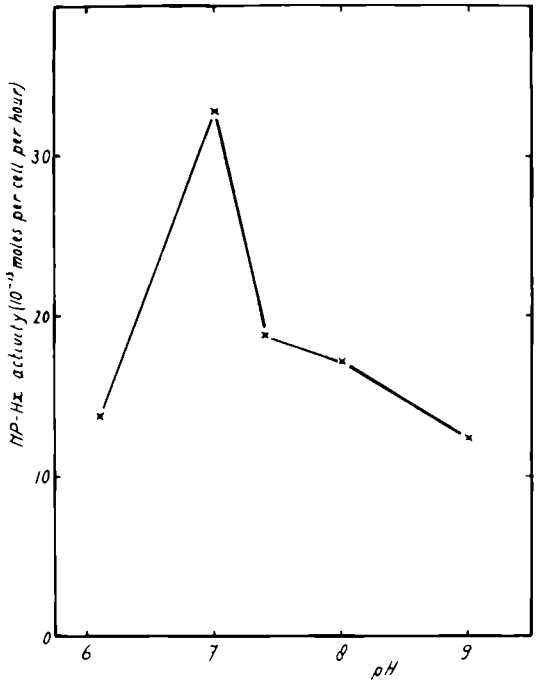

Figure 2.
pH dependence of NP-Hx from normal human fibroblasts. Each point represents an average of five measurements.

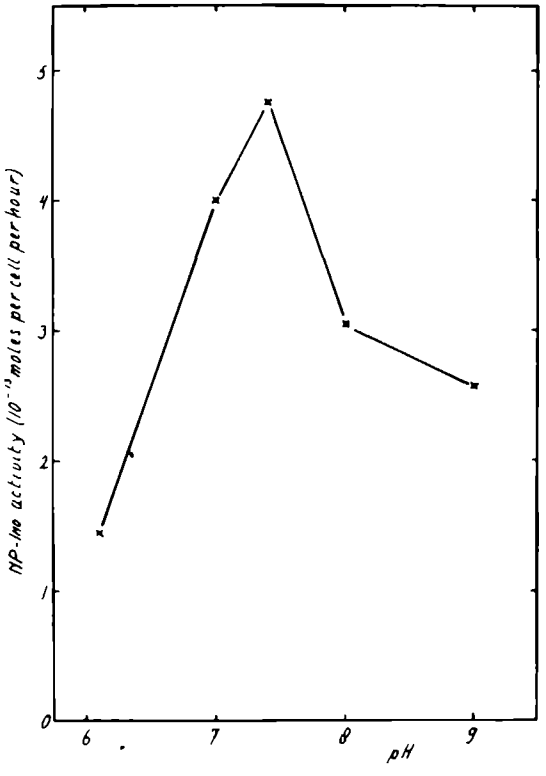

Figure 3.
pH dependence of NP-Ino from normal human fibroblasts. Each point respresents an average of five measurements.

RESULTS AND DISCUSSION

In the measurements presented here plastic leaflets cut out from the bottom of a PFD, each carrying five human fibroblasts were incubated in 0.3 µl incubation volumes in PMC's. Because enzyme activities are cell-cycle dependent, each measurement had to be done in fivefold to get a reliable average.By measuring at five different pH values, an indication of the pH optima of ADA (fig.1), NP-Hx (fig. 2) and NP-Ino (fig.3) with in total 125 cells only for each enzyme could be obtained.

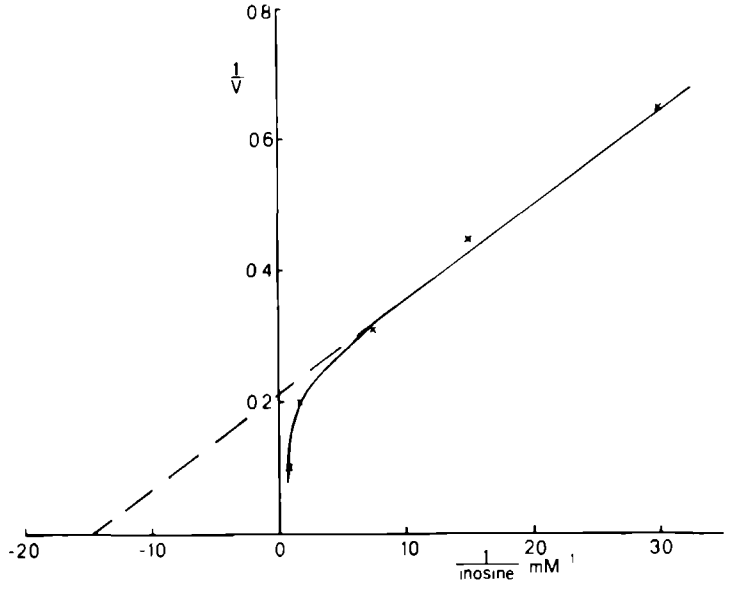

Figure 4.
Lineweaver-Burk plot of NP-Ino activity vs. inosine concentration.

The pH optima found in this way were in accordance with literature values, e.g. Agarwal et al.(7) and Meyskens and Williams (8) found a broad pH optimum, respectively, between pH 6 and 8 and between pH 5.5 and 8 for ADA from human erythrocytes. Fig.1 shows a flat peak in the same area for ADA from human fibroblasts.

By assaying at five different substrate concentrations K_M values of adenosine for ADA, of inosine for NP-Ino and of hypoxanthine and ribose-1-phosphate for NP-Hx could be determined with again only 125 cells for each K_M. The K_M of adenosine for ADA was about 33 µM. Previous studies from other groups using conventional techniques gave values of 30 µM (9), 25 µM (7) and 40 µM (8) in human erythrocytes. A K_M of inosine for NP-Ino from fibroblasts of 66 µM could be calculated. This is in good agreement with the K_M values reported for NP-Ino measured with macromethods: 58 µM (10), 67 µM (11) and 61 µM (12).

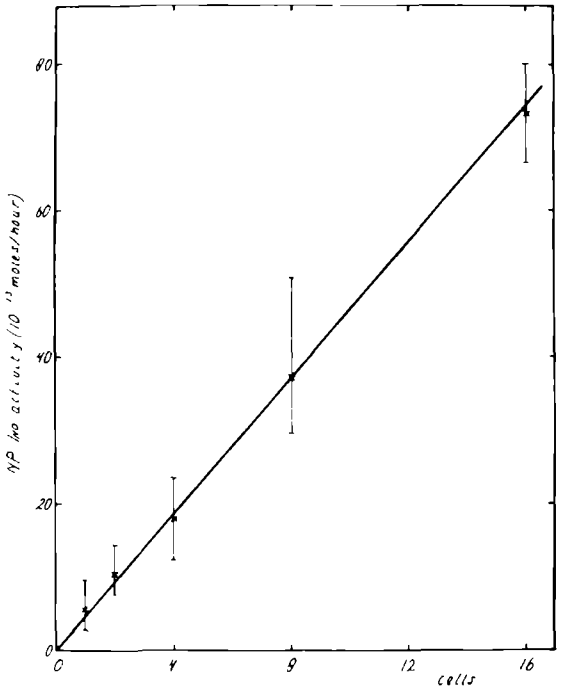

Figure 5.
Relation between NP-Ino activity and the number of cells per assay volume. Each point represents an average of ten measurements. The vertical bars indicate the range of the values measured.

In addition, the ultramicrochemical method could also detect the activation of NP-Ino at high inosine concentrations (fig.4) reported by other groups (11,12,13,14).

The K_M values of hypoxanthine and ribose-1-phosphate for NP-Hx were found to be 16 μM and 0.3 mM, respectively.

The composition of the standard reaction mixtures of NP-Hx, NP-Ino and ADA is based on the results mentioned above. Assays for NP-Hx, NP-Ino and ADA performed in this way are completely quantitative

as can be seen in fig.5 and table I. Fig.5 shows a linear dependence between the activity of NP-Ino and the number of cells incubated and table I gives ADA activity per cell per hour determined with different numbers of cells per incubation volumes and with different incubation times.

As can be seen from the comparison between the results reported here and enzyme characteristics known from literature, the method presented in this study offers a rapid and accurate way to get an indication of enzyme kinetics, consuming only an extremely little amount of cell material.

These types of ultramicrochemical enzyme characterisations are of course not restricted to the enzymes mentioned above but can be applied to any enzyme assay making use of radioactive (1) or fluorogenic substrate (15). This technique can be very useful when there is only a small number of cells available e.g. when several enzymes have to be tested in human biopsies or in biochemical research where prolonged cultivation of a cell clone to obtain more material would take too much time.

Table I.

ADA activity of human control fibroblasts, expressed in 10^{-13} moles/cell/hr., determined with 5 and 10 cell incubations using different incubation times.

number of cells per incubation volume	incubation time	
	110 min	230 min
5 cells	7.94	7.83
10 cells	8.67	not tested

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Characterisation of Purine Nucleoside Phosphorylase from Fibroblasts Using Ultra-Microchemical Methods

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Key Words Purine nucleoside phosphorylase Ultramicrochemistry Enzyme characterisation Cultured fibroblasts Immune disease

Abstract A new technique to quantitate nucleoside phosphorylase (NP) activity in single or small numbers of counted visually selected cells is presented Fibroblasts were cultivated on the plastic film bottom of culture dishes After lyophilisation *in situ* plastic film leaflets carrying a counted number of cells were cut out and tested for NP activity Some properties of NP, including temperature stability, pH optimum and substrate affinity, have been studied The data obtained suggest that NP might play a regulatory role in the purine interconversion pathway

Introduction

Purine nucleoside phosphorylase (NP, EC 2 4 2 1) catalyses the reversible reaction of the purine nucleosides inosine, xanthosine and guanosine with inorganic phosphate to ribose-4-phosphate and hypoxanthine, xanthine and guanine, respectively [KALCKAR, 1947] Because of its intriguing kinetic properties and the similarities and differences in NP from different mammalian sources the enzyme has widely been studied [KIM *et al*, 1968, AGARWAL and PARKS, 1969, AGARWAL *et al*, 1975, MURAKAMI and TSUSHIMA, 1975]

Recently several cases of NP deficiency in association with immune disease in man have been reported [GIBLETT *et al*, 1975, GRISCELLI *et al*, 1977, SIEGENBEEK VAN HEUKELOM *et al* 1977]

For both diagnostic and fundamental purposes ultra-microchemical methods offer the obvious advantage that many determinations can be car-

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ried out with a small amount of biopsy material (bone marrow and peripheral blood cells, skin, hair roots).

The main purpose of the present paper is to introduce a quantitative radiochemical NP assay (with hypoxanthine and inosine as substrates) with counted numbers of cultured human skin fibroblasts and to report on some kinetic properties of NP, such as temperature stability, pH optimum and substrate affinity, which can be studied with very limited amounts (100–200) of cells.

Materials

All chemicals were obtained from Merck (analytical grade) except ribose-1-phosphate (R-1P; Boehringer); bovine serum albumine (BSA), unlabeled hypoxanthine, inosine and IMP (Sigma); penicillin and streptomycin (Mycofarm; Delft) and ^{14}C-labeled hypoxanthine and inosine (Radiochemical Centre, Amersham). The fibroblasts were derived from forearm skin biopsies from healthy volunteers.

Methods

Fibroblasts were grown in plastic film dishes (PFDs) [Hösli, 1972] in HAM F10 medium containing 15% fetal calf serum and 100 IU/ml penicillin and 100 mg/ml streptomycin. 2 days after seeding, being in the log-growth phase, the cells were washed three times with physiological saline, shock-frozen in liquid nitrogen and lyophilized *in situ*. Plastic leaflets with counted numbers of cells were cut out free-hand from the bottom of the PFD under a stereomicroscope and these leaflets were incubated in a reaction volume of 0.3 μl in a Parafilm microcuvette (PMC), moulded in a strip of conventional parafilm [Hösli, 1972].

The incubation mixture for the NP reaction with hypoxanthine as a substrate (NP-Hx) contained 0.17 M Tris-HCl buffer (pH 7.0), 0.5‰ bovine serum albumine (BSA) to prevent surface denaturation of the enzyme, 0.8‰ penicillin, 0.8‰ streptomycin, 3.3 mM EDTA to inhibit the hypoxanthine phosphoribosyl transferase reaction, 1.7 mM phosphate and 0.133 mM 8-^{14}C hypoxanthine (spec. act. 50 mCi/mmol). For the reverse NP reaction with inosine as substrate (NP-Ino) the reaction mixture was 0.17 M Tris-HCl buffer pH (7.4), 0.5‰ (w/v) BSA, 0.8‰ penicillin, 0.8‰ streptomycin, 3.3 mM EDTA, 1.7 mM R-1-P and 0.267 mM 8-^{14}C inosine (spec. act. 50 mCi/mmol). Reactions were terminated by pushing out the total contents of the PMCs onto Whatman 3-mm paper strips. Substrate and reaction product were separated by descending chromatography [Uitendaal *et al.*, 1976]. Unlabeled reference compounds were cochromatographed to visualize after separation the purine spots under UV light. The spots were cut out and counted in a Packard 2450 liquid scintillation counter [Uitendaal *et al.*, 1976].

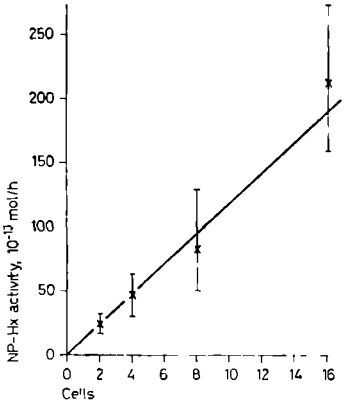

Fig. 1. **NP-Hx activities for different numbers of cells. For each point the mean and range of ten independent isolations are indicated.**

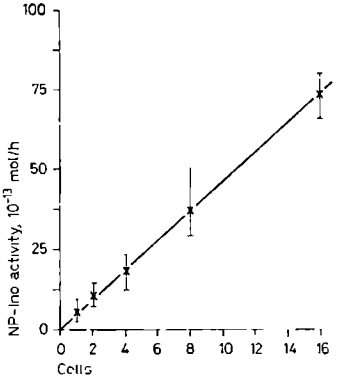

Fig. 2. NP-Ino activities for different numbers of cells. For each point the mean and range of ten independent isolations are indicated.

Results

Stability of the Enzyme

To test whether NP is stable during several hours at 37 °C, leaflets with five cells each were preincubated in a buffer, consisting of the reaction mixture

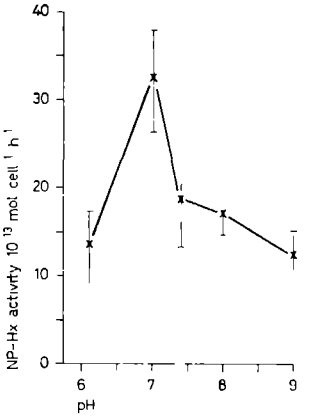

Fig 3 pH dependency of NP-Hx Each point represents a mean of five values, each measured with five cells per incubation volume The vertical bars represent the ranges of the activities found

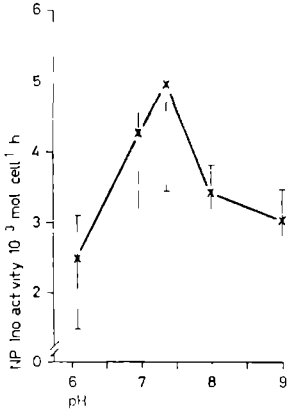

Fig 4 pH dependency of NP-Ino Each point represents a mean of five values, each measured with five cells per incubation volume The vertical bars represent the ranges of the activities found

without the radioactive substrate After different preincubation times, the substrate was added and the activities of NP-Hx and NP-Ino were tested The enzyme, measured in both directions, was found to be stable at 37 °C for at

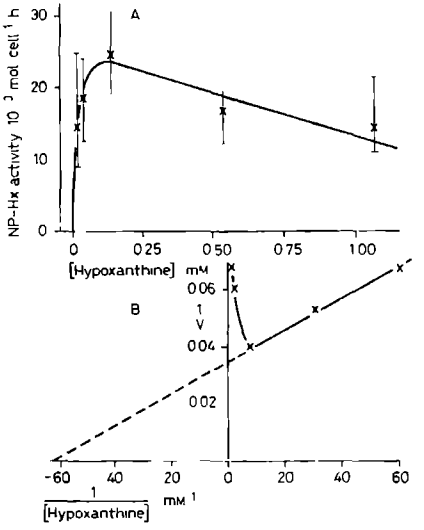

Fig 5. NP-Hx activities vs hypoxanthine concentration (A) and NP-Hx activities^{-1} vs hypoxanthine concentrations^{-1} (B, Lineweaver-Burk plot) as measured with 125 cells in total (5 cells per incubation) The bars in A indicate the ranges found

least 12 h.

Relation Between Enzyme Activity and the Number of Cells Tested

As can be seen in figures 1 and 2 there is a linear relation between the number of cells tested and the average amount of product formed.

pH Optimum

By measuring at five different pH values, incubating leaflets carrying five cells each and performing each determination five-fold, pH optimum curves for NP-Hx and NP-Ino could be obtained with, in total, 125 cells for each curve (fig. 3, 4, respectively). The optimal pH values thus obtained are pH 7.0 for the NP-Hx reaction and pH 7 4 for the NP-Ino reaction.

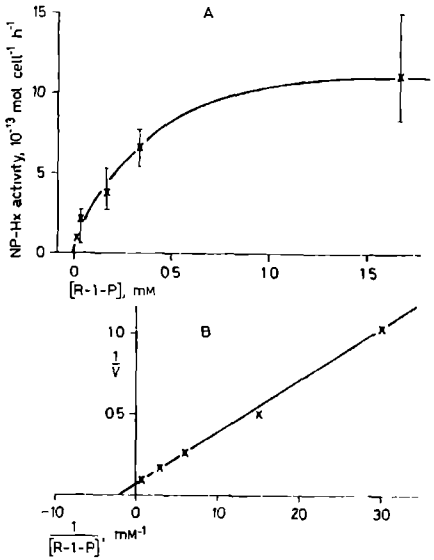

Fig.6. NP-Hx activities vs. R-1-P concentrations (A) and NP-Hx activities^{-1} vs. R-1-P concentrations^{-1} (B; Lineweaver-Burk plot) as measured with 125 cells in total (5 cells per incubation). The bars in A indicate the ranges found.

Substrate Affinity

By assaying at five different substrate or cosubstrate concentrations, it was possible to obtain K_m values for hypoxanthine and R-1-P for NP-Hx and for inosine for NP-Ino, again using 125 cells per K_m determination.

The substrate dependency curve and the Lineweaver-Burk plot for NP-Hx with varying hypoxanthine concentrations are given in figure 5. The K_m value calculated from the points with a low hypoxanthine concentration is 16 μM. There is an upward bend of the curve at high substrate concentrations, suggesting inhibition of NP-Hx by its substrate. The substrate dependency curve and Lineweaver-Burk plot of NP-Hx for R-1-P (fig. 6) allowed to calculate a K_m of NP for R-1-P of 0.30 mM. In figure 7 the Michaelis Menten curve and the Lineweaver-Burk plot of NP-Ino with inosine as a substrate are shown. Extrapolation of the linear part of the curve permitted reading of a K_m value of 66 μM inosine.

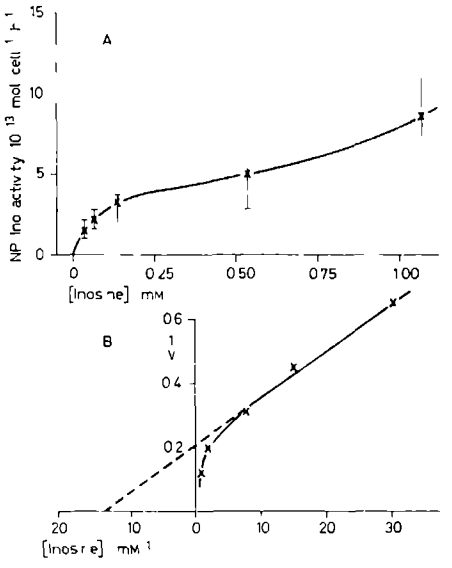

Fig 7 NP-Ino activities vs inosine concentration (A) and NP-Ino activities ^{1} vs inosine concentrations ^{1} (B, Lineweaver-Burk plot) as measured with 125 cells in total (5 cells per incubation) The bars in A indicate the ranges found

Discussion

In most studies published thus far only the NP-Ino reaction has been assayed [KALCKAR, 1947, KIM *et al*, 1968, TURNER *et al*, 1971, AGARWAI *et al*, 1975, MURAKAMI and TSUSHIMA, 1975, GIBLETT *et al*, 1975]

In the method used, the reaction product hypoxanthine is converted to uric acid by adding xanthine oxidase to the reaction mixture and uric acid formation is followed spectrophotometrically When using radioactively labeled hypoxanthine and inosine, both reactions of the enzyme can be studied In addition, the radiochemical method is more direct and avoids eventual disturbing effects caused by contamination in the xanthine oxidase preparation Since the NP-Hx reaction is the more active one in fibroblasts, it is advisable to use hypoxanthine as a substrate for the measurement and characterisation

of the NP reaction in normal and especially in mutant individuals.

Advantages of the present ultra-microchemical methods include:

(1) Only minute amounts of cell material and substrates are needed. Time consuming culturing procedures to collect enough cells for conventional measurements are avoided (e.g., in prenatal diagnosis, carrier detection studies) [HÖSLI, 1972, 1974].

(2) Enzyme activities are related to the number of cells and not to the amount of protein, the latter parameter being unreliable because of its dependence on cell culture dynamics. This is especially important in studies on gene expression, where small changes in enzyme activities have to be detectable [HÖSLI *et al.*, 1976].

(3) Single morphologically defined cells can be selected from a cell mixture. This permits to work with visually selected cell material which is essential in, e.g., prenatal diagnosis of inborn errors of metabolism [HÖSLI, 1974] and in studies on cellular interactions [UITENDAAL *et al.*, 1976].

Linearity between the numbers of cells tested and their average NP activity (fig. 1, 2) was found. The relatively large ranges in NP activities of the individual isolations are basically not due to methodological errors, but due to the fact that the cells were isolated from an asynchronously growing culture where the single cells display cell cycle dependent variations in enzyme activities. Therefore, in figures 1 and 2 the average values of ten measurements are given.

The results of the NP-Ino characterisation with the ultra-micro method are in agreement with findings reported in the literature, with respect to pH optimum (fig. 4) [KIM *et al.*, 1968; HUENEKER *et al.*, 1956] and K_m for inosine (fig. 7) [AGARWAL *et al.*, 1975; TURNER *et al.*, 1971; HUENEKER *et al.*, 1956].

No data being available about the kinetic properties for the NP-Hx reaction, comparison with literature values is not possible.

The activation of the NP-Ino reaction in human fibroblasts by high concentration of its substrate inosine (fig. 7), is also seen in human erythrocytes [AGARWAL *et al.*, 1975; TURNER *et al.*, 1971; AGARWAL and PARKS, 1971] and in cells from other species [AGARWAL *et al.*, 1975; DUHM, 1974; MURAKAMI and TSUSHIMA, 1975]. In human fibroblasts NP-Hx can be inhibited by its own substrate hypoxanthine (fig. 5). These findings indicate that nucleoside phosphorylase might have regulatory effects on the purine interconversion and purine salvage pathway.

Under the conditions reported in this paper cultured skin fibroblasts from a patient with severely impaired T cell immunity [GRISCELLI *et al*, 1977] showed a substantial reduction of NP activity to the same amount in both katalytic directions [UITENDAAL *et al*, 1977] The techniques presented in this paper will further be used in the study of the role of NP in purine metabolism of single immunocompetent cells and in the study of the relation between NP deficiency and immune disease in man

Acknowledgements

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MOLECULAR AND TISSUE SPECIFIC HETEROGENEITY IN HPRT DEFICIENCY

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Summary

In several patients with different degrees of HPRT deficiencies, the residual activities have been determined in both lysed and intact erythrocytes. No close correlation could be found between the degree of HPRT deficiency and the severity of the clinical expression. Unless HPRT activity in both intact and lysed erythrocytes was below detection level, the residual activity in intact red blood cells was higher than in lysates. Tissue specific heterogeneity was illustrated with a patient suffering from X-linked gout. Lysates from erythrocytes, leukocytes and cultured fibroblasts showed 1%, 8% and 100% of normal HPRT activity, respectively. Characterisation of the erythrocyte and fibroblast HPRT from this patient showed no kinetic abnormalities. However, there was a decreased heat stability. It is concluded that for a better understanding of the pathophysiology in HPRT deficiency studies on nucleated cells from the different tissues are needed.

Keywords

HPRT deficiency / Molecular heterogeneity / Tissue specific heterogeneity / Cultured fibroblasts / Ultramicrochemical enzyme characterisation

Deficiency for the enzyme hypoxanthine phosphoribosyl transferase (HPRT: EC 2.4.2.8) can lead to two clinically very different syndromes; the Lesch-Nyhan syndrome (Lesch and Nyhan,1964; Seegmiller et al.,1967) and X-linked gout (Kelley et al.,1967).

Although in general the enzyme deficiency in patients with the Lesch-Nyhan syndrome is more severe than in gouty patients, marked exceptions have been reported (Seegmiller, 1976; de Bruyn,1976). Residual HPRT activity in fibroblasts (Fujimoto and Seegmiller, 1970; Kelley and Meade,1976) and even in erythrocytes of some patients (Bakay and Nyhan, 1972; Arnold et al.,1972) have been found in the case of Lesch-Nyhan syndrome. On the other hand, a gouty patient without any detectable HPRT activity in erythrocyte lysate has been described (de Bruyn et al.,1973).

In neither of the two groups there is a complete correlation between the residual HPRT activity measured in cell lysates and the severity of the clinical picture.

This paper reports heterogeneity of HPRT activities in different cell types from patients with the Lesch-Nyhan syndrome and with X-linked gout. In addition, the mutant enzyme in fibroblasts from one of the gouty patients has been characterised using a quantitative ultramicromethod.

Materials and Methods

Cases: Three of the four cases with HPRT deficiency presented in this study have been described clinically previously: one patient with the Lesch-Nyhan syndrome (van der Zee, 1972; Lommen,1973) and two individuals with X-linked gout (Geerdink et al.,1973; van Herwaarden et al.,1976).

Materials: All chemicals used were purchased from Merck, analytical grade, except for bovine serum albumin, fraction V (Sigma); phosphoribosylpyrophosphate (PRPP), A grade (Calbiochem); IMP (Boehringer, Mannheim); [8-^{14}C] hypoxanthine (Radiochemical Centre, Ltd., Amersham); and penicillin and streptomycin (Mycopharm, Delft).

Incubations with erythrocyte lysates: Erythrocyte lysate was prepared by three cycles of freezing and thawing of one volume of washed erythrocytes in two volumes of 0.01 M Tris-HCl buffer (pH 7.4). Stroma was removed by 30 min. centrifugation at 30,000 g.

HPRT activity was determinee by incubating 10-20 μg of lysate protein at 37°C in a

40 µl incubation volume with 0.1 mM [8-^{14}C] hypoxanthine, 0.125 M Tris-HCl buffer (ph 7.4), 12.5 mM $MgCl_2$ and 1.25 mM PRPP, for 30-60 minutes.

Incubations with leukocyte lysates: Leukocytes were isolated from the buffy layer after centrifugation of heparinized blood for 15 min at 800 g. The buffy layer was incubated for 10 min at 37°C in a buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.4)) to lyse erythrocytes and after two additional washings with the same buffer, the leukocytes were lyophilized and solved in 0.01 M Tris-HCl buffer (pH 7.4). An amount of 5-15 µg protein was incubated at 37°C for 30 min in 40 µl of the same incubation mixture as for the erythrocyte lysate HPRT determinations.

Incubations with intact erythrocytes: HPRT activity in intact erthrocytes was determined by incubating 166 µl packed erythrocytes in a total volume of 500 µl in a buffer with 0.01 mM [8-^{14}C] hypoxanthine, 43.5 mM sodium phosphate (pH 7.4), 65.25 mM NaCl, 5.75 mM $MgCl_2$ and 10 mM glucose at 37°C. The reaction was stopped by rapidly cooling to 0°C and the cells were spun down (3 min, 1000 g). Both cell content and medium were analysed by means of high voltage electrophoresis (de Bruyn and Oei,1977)

Incubation with fibroblasts: The HPRT assay in cultured fibroblasts on ultramicrochemical scale was performed essentially as described before (de Bruyn et al.,1976) with some modifications in the reaction mixture as reported elsewhere (Uitendaal et al.,1977). HPRT determinations were done in fivefold with 10 cells per incubation volume in parafilm microcuvettes (Hösli,1977).

For the alternative determination of HPRT activity in fibroblasts on the "macro" scale the cells were lyophilized in situ in plastic Falcon flasks and dissolved in distilled water. An amount of 5-10 µg protein was incubated in a small Eppendorf reaction tube at 37°C for 30 min in a 30 µl reaction mixture as used for the ultramicro assay (Uitendaal et al.,1976).

In all assays, substrate and product of the reaction were separated by descending chromatography on 1x20 cm^2 Whattman 3MM strips with a 0.5 N ammonia, 0.05 N EDTA solution as an eluent. Quantification of radioactivity was perfofmed as described previously (de Bruyn et al.,1976; Uitendaal et al.,1976).

To exclude mycoplasma contamination the fibroblast cultures were tested before being used with the enzymatic test described by Hatanaka et al.(1975).

TABLE I. *Relative IMP formation (HPRT reaction) in erythrocytes from individuals with mutant HPRT*

Patient	Clinical picture	Reference	Relative IMP formation (%)	
			erythrocyte lysate[a]	intact erythrocytes[b]
W	Lesch-Nyhan	van der Zee,1972; Lommen,1973	<0.05	<0.1
S	X-linked gout	Geerdink *et al.*,1973	<0.05	<0.1
F	X-linked gout	van Herwaarden *et al.*,1976	1.00	10.1
G	X-linked gout		13.00	100.0

[a] 100% activity corresponds to 76.1 nmoles/mg protein.hr (n=9).
[b] 100% activity corresponds to 19 nmoles/ml packed cells.15 min (n=9).

Results

Molecular and tissue specific heterogeneity

No residual activity of the mutant HPRT was observed in a Lesch-Nyhan patient and in one of the gouty patients, both in erythrocyte lysate and in intact erythrocytes (Table I). In the other two gouty patients the intact erythrocytes showed a higher residual HPRT activity than the lysates: in patient G even a normal incorporation of hypoxanthine was found (Table I).

Discordance of the severity of the enzyme deficiency in different celltypes is illustrated in Table II, where the residual activities in lysates of erythrocytes, leukocytes and fibroblasts of one of the gouty patients are given.

TABLE II. *Specific HPRT activity in lysates from different cell types of patient F with X-linked gout*

Cell type	Specific activity (nmoles/mg protein.hr)		
	Control	Patient F	(% of control)
Erythrocyte	76.1	0.8	(1)
Leukocyte	107.2	13.4	(8)
Fibroblast	199	222	(100)

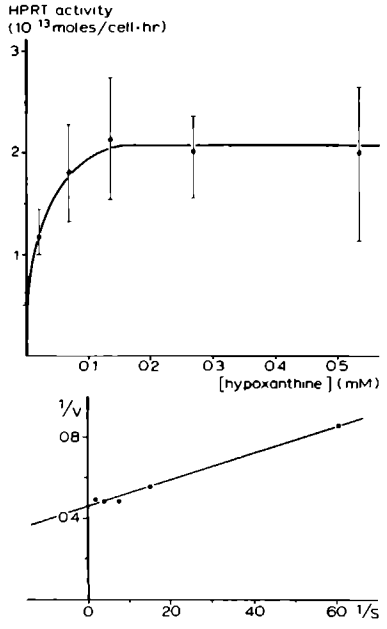

Fig. 1. Hypoxanthine dependent activity of normal human fibroblast HPRT studied with the ultramicrochemical assay. In the upper part the Michaelis-Menten curve is shown. Each point represents the mean of five individual measurements with 10 asynchronously growing, randomly selected cells each. The vertical bars indicate the actual range, which is caused by methodological errors and especially biochemical fluctuation (cell cycle). In the lower part the Lineweaver-Burk plot is shown.

Ultramicrochemical characterisation of normal and mutant fibroblast HPRT

Figures 1 and 2 give the dependence of HPRT activity in normal fibroblasts on the concentration of the substrate hypoxanthine and the cosubstrate PRPP, respectively. The Km values calculated from the linear parts of the double reciprocal plots are 11 μM for hypoxanthine and 160 μM for PRPP ($MgCl_2$ concentration 17 mM).

A conspicuous phenomenon was observed when the products of the HPRT reaction at different PRPP concentrations were analysed: the IMP formed by the HPRT reaction is often degraded to inosine, due to the action of 5'-nucleotidase. At higher PRPP concentration decreasing amounts of [8-^{14}C] inosine were detected in the reaction mixture (fig.3).

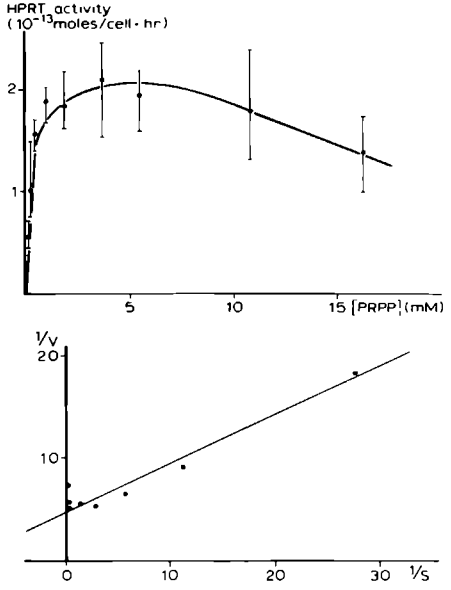

Fig. 2. Phosphoribosylpyrophosphate (PRPP) dependent activity of normal human fibroblast HPRT with the ultramicrochemical assay. In the upper part the Michaelis-Menten curve is shown. Each point represents the mean of five individual measurements with 10 cells each. The vertical bars indicate the actual range (see also legend fig.1). In the lower part the Lineweaver-Burk plot is shown.

The normal levels of HPRT activity in fibroblasts of patient F offered an excellent opportunity to characterize the mutant enzyme. The Km values for hypoxanthine and PRPP and the sensitivity for the inhibitory effect of 5mM unlabeled IMP were comparable with he values obtained for normal fibroblast HPRT (Table III).

Heat stability of normal and mutant fibroblast HPRT

By preincubating dissolved lyophilized normal and mutant fibroblasts in Eppendorf reaction tubes containing a solution consisting of the normal reaction mixture without PRPP and hypoxanthine at 80°C for various minutes, the heat stability of normal and mutant HPRT could be compared. The results are given in fig.4. After one minute preincubation at 80°C hardly any activity of the mutant HPRT could be detected, whereas even

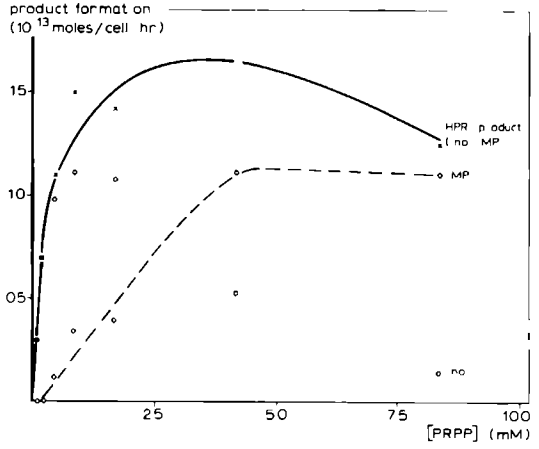

Fig 3 The effect of phosphoribosylpyrophosphate (PRPP) concentration on the degradation of the HPRT reaction product IMP to inosine (Ino) studied with the ultramicrochemical assay Each value represents the mean of five individual measurements with 10 cells each For the sake of clarity the ranges are not indicated in this figure ✕, total product formation (IMP + Ino) ◇, IMP recovered ○, IMP degraded to inosine

TABLE III. *Characteristics of HPRT in fibroblast and erythrocyte lysate from a normal individual and from patient F with X-linked gout*

Characteristics	Fibroblast lysates		Erythrocyte lysates	
	Control	Patient F	Control	Patient F
Vmax	2 28[a]	2.17[a]	88 8[b]	0 83[b]
Km hypoxanthine	11 μM	14 μM	8 μM	17 μM
PRPP	0 18 mM	0.24 mM	0 07 mM	0.24 mM
Inhibition by 5 mM IMP	52%	53%	n.t.[c]	n t [c]
Activity after 4 min 80°C	39%	0%	37%	19%

[a] Activity expressed in 10^{-13} moles/cell hr
[b] Activity expressed in nmoles/mg protein.hr
[c] n t Not tested

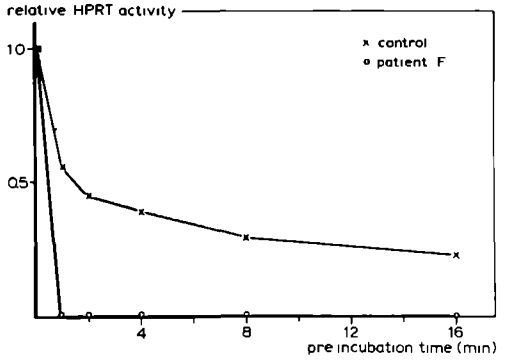

Fig. 4. Thermostability at 80°C of fibroblast HPRT from a control and from an individual with a mutant HPRT (patient F), studied with the HPRT assay on the "macro"-scale (see also under *Results*). ✕ , control HPRT. ○ , mutant HPRT (patient F).

after 16 minutes still 20% of the normal HPRT activity was present. The presence of 7 mM PRPP during the preincubation did not result in an increase of the heat stability neither for normal nor for mutant enzyme under the conditions used (data not shown).

Heat stability was also tested at 37°C. The enzyme activity after preincubation at 37°C in the presence and absence of PRPP are depicted in figs.5 and 6. The mutant HPRT was less stable than normal HPRT. In contrast to the findings at 80°C PRPP (7 mM) stabilized both mutant and normal HPRT at 37°C.

Properties of normal and mutant erythrocyte HPRT

The mutant enzyme in erythrocyte lysate could also be characterized with respect to substrate affinity and heat stability. The results are summarized in Table III and essentially confirm the results obtained for the fibroblast HPRT. In contrast with the findings in fibroblasts both mutant and normal HPRT were stabilized by the presence of PRPP during preincubation at 80°C (Table IV).

Discussion

Table I illustrates the lack of correlation between residual HPRT activities in red cells and the clinical expression of the enzyme deficiency. Not only similar biochemi-

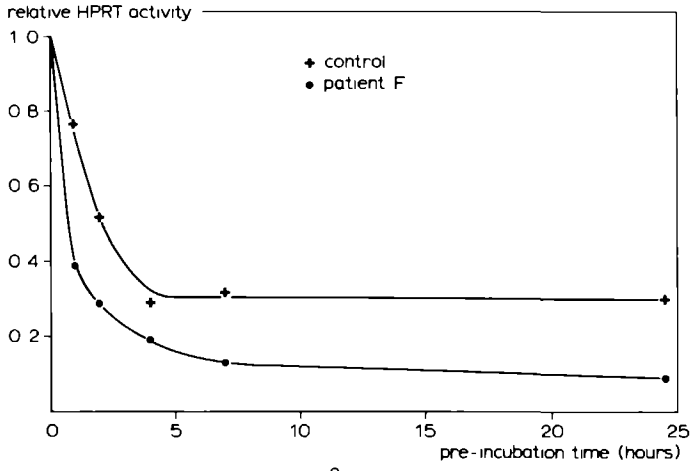

Fig. 5. Thermostability at 37°C of fibroblast HPRT from a control and from an individual with a mutant HPRT (patient F), studied with the HPRT assay on the "macro"-scale (see also under *Results*).+, control HPRT.● , mutant HPRT (patient F).

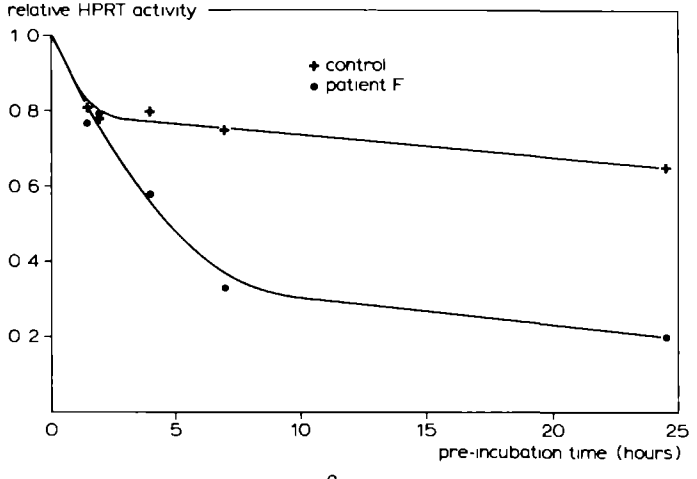

Fig. 6. Thermostability at 37°C of fibroblast HPRT from a control and from an individual with a mutant HPRT (patient F), in the presence of 7 mM phosphoribosylpyrophosphate as studied with the HPRT assay on the "macro"-scale (see also under *Results*). +, control HPRT.● , mutant HPRT (patient F).

TABLE IV *Heat stability at 80°C of HPRT in erythrocyte lysate from a normal individual and from patient F with X-linked gout*

Preincubation time (min)	Remaining activity (percentage)			
	no PRPP		5mM PRPP	
	Control	Patient F	Control	Patient F
0	100	100	100	100
4	37	19	57	27
8	20	9	46	23

cal results were obtained with blood from the patients W and G, who present a different clincal picture, but also marked differences in residual HPRT activities between the three patients suffering from X-linked gout were observed

In general, Lesch-Nyhan patients show HPRT activities below detection level in their red blood cell lysates, while a less severe deficiency for HPRT is associated with gout However, this correlation is not entirely consistent. Lesch-Nyhan patients sometimes have residual activities (Bakay and Nyhan,1972, Arnold et al.,1972) up to 34% under certain conditions, although under more physiological conditions the residual activity was considerably lower (McDonald and Kelley,1971) On the other hand, an adult gouty patient has been described without any demonstrable HPRT activity in red cell lysates (patient S)(Table I) Further clinical and biochemical data are presented by Geerdink et al. (1973) and de Bruyn et al. (1973). In leukocytes and fibroblasts no significant activity was detected in this patient. Another gouty patient without any demonstrable erythrocyte HPRT activity showed 10-15% residual activity in leukocyte lysate (Dancis et al.,1973) This case resembles more our patient F (Table II) The full range of residual HPRT activities now goes from undetectable for patient S, through 30% (Yu et al.,1972) and 60% (Becker and Sweetman,1975) to 100% (Benke and Herrick,1972) in red cell lysates from patients with X-linked gout Therefore, it seems very likely that there is a broad range in HPRT mutants, though the different methods used by different authors might simulate molecular heterogeneity.

The tissue specific heterogeneity in HPRT deficiency is best illustrated in our patient F. Whereas in erythrocyte lysate only a low residual HPRT activity was found

(1%), lysate from cultured fibroblasts displayed an HPRT activity comparable to normal (Table II). Mycoplasma as a source for the HPRT activity has been excluded (cfr. materials and methods).

The HPRT activity from the gouty patient with 100% activity in red blood cell lysates (Benke and Herrick,1972) was shown to be a Km mutant. With respect to the parameters tested in the present study, the mutant fibroblast HPRT from patient F differs from normal fibroblast HPRT only in heat stability (Table III): even after a preincubation of only one minute at 80°C virtually no activity of the mutant enzyme could be detected (fig.4). Addition of 7 mM PRPP before heating did not increase heat stability neither of the mutant, nor of the normal enzyme. The heat inactivation experiments with fibroblast HPRT were repeated at a lower temperature (37°C) to follow the inactivation process in more detail. Again, the normal HPRT was more stable than the mutant enzyme. In contrast to the results obtained after preincubation at 80°C, a stabilizing effect of PRPP was observed on both normal and mutant fibroblast HPRT. The normal enzyme, however, was protected to a higher degree than the mutant enzyme. In erythrocyte lysate a protecting effect of PRPP could be demonstrated even at 80°C for both the mutant and the normal HPRT (Table IV). This is in agreement with findings in the literature for normal (McDonald and Kelley,1971; Sperling et al.,1972) and mutant (Sperling et al, 1972) HPRT in erythrocytes. The difference in heat stability at 80°C noted between erythrocyte lysates and fibroblast lysates are most likely due to the lower protein concentration in the fibroblast lysate during preincubation (approximately a factor 250) Protein concentration is known to have a marked effect on the HPRT stability in fibroblast lysates even at lower temperatures than those applied in the present study (Zoref et al.,1974).

The ultramicrotechnique used to study the kinetics of fibroblast HPRT of patient F has previously been shown to be useful in kinetic studies of other enzymes of purine metabolism (Uitendaal et al.,1977). The reasons for using ultramicromethods have been discussed in detail elsewhere (Hösli,1974; Hösli,1977; Hösli and de Bruyn,1977). It should be noticed that, in contrast to conventional procedures, in which cultured fibroblasts are trypsinised and disrupted by sonication or freezing and thawing, in the present method the growing fibroblasts are lyophilized *in situ* (Hösli,1972), resulting in higher specific HPRT activities (unpublished observations). This is especially important when preparing cell lysates from cell strains that have a reduced enzyme stability.

Kinetic data thus obtained with fibroblasts are comparable with those found with

erythrocytes (Table III) and those reported in the literature (Henderson et al.,1968; McDonald and Kelley,1971).

It is well known that the product of the HPRT reaction, IMP, is often dephosphorylated to inosine as a result of the action of 5'-nucleotidase. Several nucleotidase inhibitors have been proposed (Murray and Friedrichs,1969; Fox and Marchant,1977). However, in our hands these compounds failed to exert a significant inhibitory effect on 5'-nucleotidase. The present finding that PRPP in concentrations exeeding 5 mM prevents extensive IMP degradation (fig.3) might provide a solution for this problem. This is of special interest for HPRT assays on cells other than erythrocytes, based on the measurement of IMP only (Harris and Cook,1969; Chow et al.,1970), since without inhibition of 5'-nucleotidase an underestimation of the HPRT activity is obtained.

The present study on different cases of HPRT deficiency underlines that there is no simple correlation between the clinical picture and the enzyme activity measured in vitro, but that the symptomatology in patients has to be explained by a more complex molecular and tissue specific heterogeneity of HPRT mutants.

In addition, it seems to be particularly helpful to include nucleated cells from different tissues to elucidate the pathophysiology of HPRT deficiency.

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FLUCTUATING ADENOSINE DEAMINASE ACTIVITIES IN CULTURED FIBROBLASTS

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Because it has attracted the attention of many workers in the field of physiology (1), genetics (2), hematology (3), immunology (4-6) and oncology (7-11), adenosine deaminase (ADA; EC 3.5.4.4) seems to occupy a key position in purine metabolism.

Erythrocytes can be used for practical diagnosis of ADA deficiency in severe combined immune deficiency (SCID). On the other hand, cultured cells are necessary for prenatal diagnosis of ADA deficiencies and are more suitable as a model system for the analysis of the central role of ADA in purine metabolism.

Considerable variation in specific activities of ADA has been described in normal cultured fibroblasts (12,13). Therefore, a sensitive and reproducible radiochemical ultramicromethod has been developed which permits to quantitatively express ADA activity per cell and not per protein. This helps to reduce methodological errors in regulatory studies and in prenatal diagnosis of ADA deficiencies.

Materials and Methods

All chemicals were obtained from Merck (analytical grade) except for bovine serum albumine, fraction V, (Sigma) and [8-^{14}C] labeled adenosine (Radiochemical Centre, Amersham; 49 mCi/mmole).

The equipment and techniques for the ultramicrochemical measurements making use of Plastic Film Dishes (PFD's) and Parafilm Micro Cuvettes (PMC's) have been described

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previously (14,15).

Normal diploid skin derived fibroblasts are grown on the plastic bottom of a PFD. After lyophilisation of the cells, small leaflets, carrying a counted number of visually selected fibroblasts (5 or 10), are cut out from the plastic bottom of the PFD under a stereomicroscope. This is done in a climatised room (18°C; 30-40% relative humidity). These leaflets are incubated in PMC's in a 0.3 μl incubation mixture containing 0.17 mM Tris-HCl buffer (pH 7.4), 0.5 mg/ml bovine serum albumine to prevent surface denaturation of the enzyme, 0.8 mg/ml penicillin, 0.8 mg/ml streptomycin and 0.33 mM 8-^{14}C labeled adenosine (representing approximately 9,000 cpm).

The reaction was allowed to proceed at 37°C for 90-120 minutes. During this incubation time approximately 5-15% of the substrate was deaminated. To stop the reaction the PMC's were uncovered and placed upside down on DEAE paper strips (Whatman DE 81; 1x20 cm^2) and the contents were squeezed out with the rounded bottom of an Eppendorf cuvette. In this way less than 3% of the original content remained in the PMC's. Product and substrate were separated by descending chromatography with 1 mM ammonium formate. The substrate adenosine (R_f= 0.54) and the product inosine (R_f= 0.13) and its degradation product hypoxanthine (R_f= 0.08) were cut out from the strips and counted in a liquid scintillation counter with an efficiency of 90%.

AMP formation by adenosine kinase did not occur to a measurable extend, probably because the endogenous ATP content of the cells on each leaflet (maximally 10 cells) was not sufficient to allow the kinase reaction.

Results

To show the relation between the number of cells incubated and the amount of product formed, Table 1 summarizes the results of measurements with 5 or 10 fibroblasts cut out from the same culture dish. Product formation is linear with time for at least four hours under the conditions described (data not shown).

To investigate whether the method is also reproducible in a series of subsequent ADA measurements in cells from the same culture dish, fibroblasts were lyophilised and stored in a dry atmosphere (silicagel) at 18°C in a climatised room up to one week. On subsequent days five leaflets, carrying five cells each, were cut out and assayed for ADA activity. Fig. 1. shows the mean and the real range of the measurements. The ADA activities remain stable during one week at 18°C under the present conditions.

ADA activities in different passages of the same control strain repeatedly showed

TABLE 1

ADA activities of human fibroblasts isolated from the same culture dish (PFD). Incubation: 110 min at 37°C

Number of cells per incubation	Product measured (cpm)	Enzyme activity[a] (10^{-13} moles/cell.hr)
5 cells	477	6.98
"	352	5.07
"	552	8.52
"	599	8.86
"	378	4.98
mean (± S.D.)		6.88 (± 1.83)
10 cells	1057	8.45
"	859	6.58
"	814	6.33
"	971	7.81
"	829	6.59
mean (± S.D.)		7.15 (± 0.93)

[a]The specific enzyme activity is calculated from the net product formed and is expressed in 10^{-13} moles/cell.hr. The total input of radioactive substrate slightly varies for the individual incubations; this is due to micropipetting errors. There is some contamination (approximately 1%, varying from batch to batch) of substrate with product. To correct for this, the error in each individual measurement is calculated from the sum of product and remaining substrate. Therefore, the product measured in cpm is not directly representative of the product actually formed by the enzyme reaction.

considerable fluctuations. An example of this is given in fig. 2.

Discussion

Considerable variation in ADA levels of normal human fibroblasts has been reported by other investigators, as is illustrated by Table 2. To reduce the methodological errors inherent to the conventional "macro" ADA determinations, we have chosen for a relatively simple ultramicrochemical approach (14,15) for three major reasons: 1. In

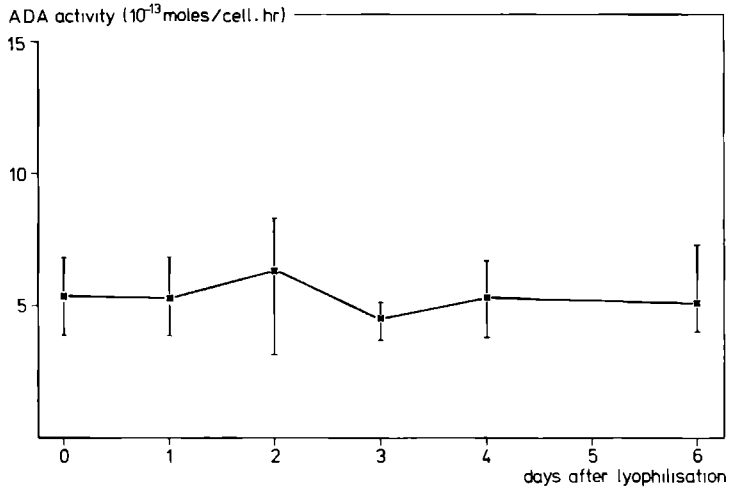

Fig. 1. ADA activity of normal human fibroblasts from the same culture dish after different times of storage at 18°C in a dry atmosphere. Each point represents the mean of five determinations with five cells each. The vertical bars indicate the actual range of the measurements.

TABLE 2

ADA activities in cultured fibroblasts as reported from different laboratories

Numbers of cell strains tested	Source	Mean ADA activity[a]	S.D.	S.D. as percentage of the mean	Reference
8	skin	757	210	28%	(27)
26	skin	876	406	45%	(12)
20	amniotic fluid	858	402	46%	(„)
15	amniotic fluid[b]	1,032	467	45%	(28)
17	amniotic fluid[b]	1,140	474	42%	(„)
9	amniotic fluid[b]	1,554	360	23%	(13)
9	amniotic fluid[b]	2,141	437	20%	(„)

[a] ADA activity is expressed in nmoles of product formed/mg protein.hr.
[b] Assayed with two different methods.

studies of ADA activities reported thus far the activities have always been determined in trypsinised cells that were disrupted by sonication or freezing and thawing. Trypsinisation causes an uncontrolable loss of cell protein, leading to erroneous specific activities. 2. As compared to lyophilisation, sonication or freezing and thawing affects the activity of several enzymes of purine metabolism, including ADA, hypoxanthine-guanine phosphoribosyl transferase and purine nucleoside phosphorylase (unpublished observations). The adversary treatments mentioned under 1 and 2 are avoided in the present method: the growing cells are lyophilised *in situ*. 3. In the ultramicrochemical method the enzyme activity is related to the exact number of cells used in the assay and not to the amount of protein, which itself is subject to fluctuations due to cell culture dynamics.

The present ultramicrochemical ADA assay is quantitative and reproducible as is

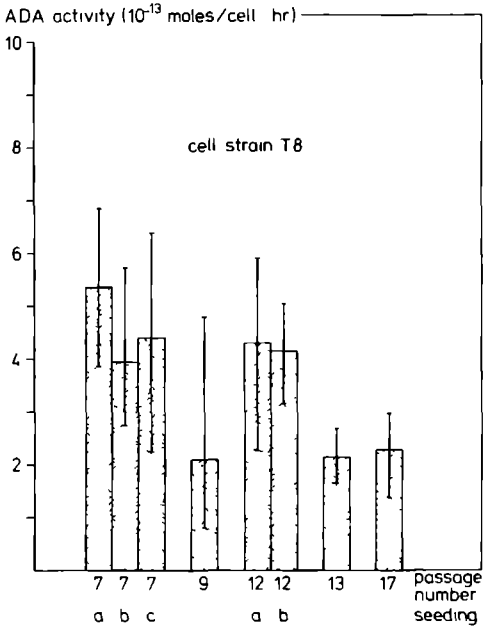

Fig. 2. ADA activity of different passages and parallel seedings of a human control fibroblast strain. Each block represents the mean of five determinations with five cells each. The vertical bars indicate the actual range of the measurements.

shown by the results in Table 1. The reproducibility is confimed by the results presented in fig.1: the mean activities of measurements in fivefold on several consecutive days on one and the same culture show no essential variation. The actual range of the measurements are relatively large. This has to be accepted if ultramicrochemical measurements are carried out with only five cells isolated on a plastic film leaflet from asynchronously growing fibroblast cultures. Another conclusion that can be drawn from fig.1 is, that ADA is stable for at least a week under the conditions used. This is an additional advantage of the lyophylisation procedure and subsequent storage at 18°C with a desiccant: it has been reported that ADA activity rapidly decreases upon storage in aqueous solutions at -20°C (16,17).

In spite of the reproducibility of our assay considerable fluctuations in ADA activity within cell strains were noted: an example is given in fig.2. This could also be demonstrated statistically when the results of the individual measurements were subjected to variance analysis (Table 3). The variance of the mean activities of different passages of one cell strain (variance between the populations compared) was significantly different from the variance introduced by the method (variance within the populations compared). Obviously the population of ADA activities of the cells from dif-

TABLE 3

Variance analysis of ADA determinations

Comparison	Variance ratio[a]	Degrees of freedom		Significance[b]	Conclusion
		within	between		
Passages:					
pass.7-9-12-13-17	4.42	29	7	s.	heterogeneous
Seedings:					
pass.7, seeding a-b-c	1.54	14	2	n.s.	homogeneous
pass.12,seeding a-b	53.01	7	1	n.s.	homogeneous
Storage times:					
pass.7, seeding a	1.29	24	5	n.s.	homogeneous

[a]Variance ratio: $\frac{\text{Variance between the populations of determinations}}{\text{Variance within the populations of determinations}}$

[b]Significance is derived from Snedecor's F test:
s.: significant difference: $p<0.01$
n.s.: no significant difference: $p>0.01$

ferent passages of the same cell strain cannot be considered to form a homogeneous ADA activity population (Table 3). Although culture conditions were kept as constant as possible with respect to medium, serum, pCO_2, cell density, time of harvesting etc., it was not possible to eliminate the factors that are responsible for the fluctuations in ADA activity in a genetically homogeneous population. Repeated tests for mycoplasma using both microbiological and enzymological (29) techniques were negative. This last test cannot detect M. pneumoniae, that has no ADA activity, and M. lipophilium, that has ADA activity (29). Especially this last type of mycoplasma, however, is very rare as cell culture contaminant and requires a very lipid-rich medium, which was not the case in our experiments. Therefore, our conclusion is, that mycoplasma contamination can be excluded as an explanation for the observed fluctuation in ADA activity. This conclusion is confirmed by the negative results of the microbiological assay.

Cells of one seeding, all cut out from the same culture dish, constitute a statistically homogeneous population. This provides additional support for the conclusion that the present ultramicromethod is reproducible (Table 3).

Human fibroblasts often contain, apart from the three or four isozymes found in red blood cells (RBC-ADA), an additional electrophoretically different "tissue-specific" ADA isozyme (18,19). The specific activity of the low molecular weight RBC-ADA appeared to be 20 times higher than that of the high molecular weight "tissue" ADA (16). A "conversion factor" has been detected that converts RBC-ADA to "tissue" ADA (20-22). In cultured fibroblasts the ratio between RBC-ADA and "tissue" ADA can vary from almost no RBC-ADA to only RBC-ADA from cell strain to cell strain (13,17) and even within one cell strain (19). Edwards *et al.* (19) reported fluctuations in the relative "tissue" ADA content when testing different culturing and harvesting conditions for the fibroblasts, but no systematic correlation was revealed. Concanavalin A stimulated lymphocytes showed increased ADA activities concomittant with a shift from "tissue" to RBC-ADA isozymes (23,24). The more stable high molecular wieght "tissue" ADA could be a "storage form" of the enzyme, which can rapidly be converted into the low molecular weight, more active form. Studies are in progress to investigate whether a correlation exists between the isozyme patterns and the ADA activity and to identify conditions which could cause rapid shifts from high- to low-molecular weight form. In addition, these forms can be characterised ultramicrochemically using the same methods that have been reported for some other enzymes of purine metabolism (25,26).

In patients with SCID associated with ADA deficiency fibroblastic residual ADA activities up to 20% of normal have been observed (13). In view of the wide margins of

control ADA activities heterozygote detection in cultured fibroblasts has turned out to be very difficult (12,13,27). In prenatal diagnosis normal, heterozygous and mutant cells must be clearly discriminated. Even with the present ultramincrotechniques - which have the advantage to express enzyme activities per cell and permit to analyse very small numbers of morphologically defined amniotic fluid cells - it seems difficult to definitively establish such a prenatal diagnosis. Therefore, the mechanisms underlying the strong fluctuations in ADA activity should first be experimentally elucidated.

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Summary

A sensitive and reproducible ultramicro assay for adenosine deaminase (ADA; EC 3.5.4.4) in cultured human fibroblasts is presented. The assay is based upon the incubation of a counted number of lyophilized cells (5 or 10) with a mixture containing [8-^{14}C] adenosine.

The mean ADA activity of desiccated lyophilized fibroblasts from the same seeding remained constant for at least one week, when stored at 18^{o}C. The mean fibroblastic ADA activities from control strains displayed considerable fluctuations in different passages. Because culture conditions were kept identical, the fluctuations were attributed to biological rather than methodological variations.

It was concluded that even with a sensitive quantitative measurement of ADA activity in amniotic fluid derived fibriblasts it might be very difficult to discriminate between normal, heterozygous and ADA deficient foetuses.

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AN ULTRAMICROCHEMICAL TEST FOR MYCOPLASMA CONTAMINATION OF CULTURED CELLS

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Summary

A sensitive ultramicrochemical enzyme test for mycoplasma contamination of cultured cells is described, based on the determination of the activity of adenosine phosphorylase. The test is performed by assaying the enzymatic conversion of [8-^{14}C]-adenine and ribose-1-phosphate to [8-^{14}C]-adnosine by incubating a plastic leaflet carrying a counted number of cells (1 to 10). These leaflets are isolated from the bottom of the same Plastic Film Dish, in which cells are cultured for experimental or diagnostic purposes, like prenatal diagnosis of inborn errors of metabolism.

The present test should be several thousand fold more sensitive than the originally reported enzymatic method. This because 1. the adenosine phosphorylase reaction is measured in the nucleoside forming direction, which is by far the most active, and 2. the assay is performed with the cells and not with the culture medium. The latter is of special importance for the detection of low grade contaminations, where most of the mycoplasma particles are attached to cell membranes.

Key words: *mycoplasma / adenosine phosphorylase / ultramicrochemical enzyme tests / cell cultures*

Introduction

Mycoplasmas are common contaminants of cell cultures. It was e.g. in one survey reported that up to 60% of the cell strains tested were contaminated (1). Mycoplasma can have profound effects on host cells regarding nucleic acid metabolism (2,3), chromo-

somal abnormalities (4), drug response (5,6), and viral yield (1), because mycoplasmal enzymes interfere with host cell activities, such as thymidine kinase, ribonuclease and deoxiribonuclease (3), pyrimidine nucleoside phosphorylase (7), adenosine phosphorylase and adenosine deaminase (8), hexosaminidase A and adenine phosphoribosyl transferase (9) and hypoxanthine phosphoribosyl transferase (6,9).

Many experimental or diagnostic procedures require absolutely mycoplasma free cell cultures, e.g. biochemical selection for chromosome mapping (6,10) or for prenatal diagnosis of inborn errors of metabolism (9).

The mycoplasma species that most commonly contaminate cell cultures exhibit considerable activity of adenosine phosphorylase (8). This enzyme is a purine nucleoside phosphorylase (PNP) which catalyses the phosphorylysis of adenosine to adenine (PNP-Ado) and the reverse reaction (PNP-Ad). Very low activities of this enzyme have been reported to occur in mammalian cells (11,12,13) although its physiological significance is questioned (14).

In the present study a sensitive radiochemical ultramicroassay for adenosine phosphorylase has been used, measuring both catalytic directions. The test can be carried out with very small numbers of lyophilised cells. These cells can be isolated from the same culture dish that is used for experimental or diagnostic purposes.

Materials and Methods

All chemicals used were purchased from Merck, analytical grade, except for bovine serum albumine fraction V (Sigma); phosphoribosylpyrophosphate, A grade (Calbiochem); [8-^{14}C]-hypoxanthine, [8-^{14}C]-adenine and [2-^{3}H]-adenosine (Radiochemical Centre, Amersham), ribose-1phosphate (Boehringer) and penicillin and streptomycin (Mycofarm, Delft).

Skin derived fibroblast cultures were established from patients with the Lesch-Nyhan syndrome, with X-linked gout and from normal healthy volunteers. Part of the mutant cell strains was obtained from the Human Genetic Mutant Cell Repository, Camden, USA (GM 29; GM 68) and the Repository for Mutant Human Cell Strains, Montreal, Canada (GL).

The methods to culture fibroblasts in the Plastic Film Dish (PFD) have been described previously (15,16). To test the culture medium on the presence of mycoplasma, the method of Hatanaka *et al.* (8), based upon the PNP-Ado reaction, has been used: 100 μl of medium, that had been in contact with the cells for four days, were incubated with

TABLE I

HPRT and PNP activities of several uncontaminated and mycoplasma contaminated fibroblast strains from HPRT deficient individuals

Cell strain	HPRT activity (10^{-13} moles/cell.hr)[a]		Mycoplasma in the medium[b]	Cell associated PNP activity[c]	
	before contamination	after contamination		adenosine as substrate	adenine as substrate
GM 29	n.d.[d]	8.03	+	+	+
GM 68	0.01	0.02	-	-	-
GL	n.d.[d]	1.56	+	+	+
LAD 113	0.01	0.03	-	-	-
Ste	0.01	2.27	+	+	+
Ste (other subline)	0.01	0.03	-	-	-
Sto	0.29	2.98	+	+	+
WC 394	n.d.[d]	3.62	+	+	+

[a] Mean normal value 1.69 x 10^{-13} moles/cell.hr (range 1.38-2.42)

[b] Mycoplasma test was performed according to the method of Hatanaka *et al.*(8) after abnormal HPRT activities in the fibroblasts were found. + : PNP-Ado activity measured.

[c] PNP-Ado and PNP-Ad was tested in cells from the same dish that was used for the HPRT determination after contamination. + : activity present; - : activity not demonstrable.

[d] n.d. = not determined. Cells from cell banks in Camden and Montreal from male patients with the Lesch-Nyhan syndrome. HPRT activity not determined in our laboratory in uncontaminated cells.

10 µl of phosphate buffered saline containing 1 µM [2-^{3}H]-adenosine at 37°C for 20 min. The ultramicrochemical mycoplasma test on cells is based upon the use of the Parafilm Micro Cuvette (PMC) (15,17). In short, the cells to be tested are grown in a PFD, lyophilised and small plastic leaflets carrying a counted number of cells (1 to 10) are dissected from the plastic film bottom under a stereomicroscope. These leaflets are transferred into PMC's containing 0.3 µl of reaction mixture each. The PMC's are sealed and incubated in a waterbath at 37°C (17). For the PNP-Ado reaction the incubation mixture contains 10 cells in 0.3 µl of PBS containing 1 µM [2-^{3}H]-adenosine; incubation time is 2 hours. For the PNP-Ad reaction the incubation mixture contains 1 cell in 0.3 µl of 0.166 M Tris-Hcl buffer (pH 7.4) with 0.017 M EDTA; 1.7 mM ribose-1-phosphate and 0.27 mM [8-^{14}C]-adenine. Incubation time is 2 h.

Analysis of the reaction mixture is performed in two ways: 1. substrate and product are separated on Whatman 3MM paper after pushing out the complete content of each PMC. A 0.5 N ammonia, 0.05 N EDTA solution is used as an eluent. After identification of the labeled spots with the aid of reference compounds, the radioactivity is quantified in a liquid scintillation counter (Intertechnique). The results obtained in this way are given in tables I and II. 2. The contents of the PMC's are brought onto cellulose thin layer plates (20x20 cm^{2}) at 1 cm distance and ascending chromatography is performed using a mixture of n-butanol/methanol/water/concentrated ammonia (60/20/20/1; v/v). The radioactive spots are localised and quantified under a newly developed radiochromatogram reader (81)(Chromelec, model 101; Numelec, France). Photographs are taken with a polaroid camera from the data display screen. The results obtained in this way are shown in figs. 1 and 2.

The method for the ultramicrochemical quantification of HPRT activities in a small number of counted cells has been published previously (19,20)

Results

Table I summarises a study on eight fibroblast cultures from seven individuals known to be deficient for HPRT (first column). Column 2 shows that five subcultures out of these eight displayed at a later period considerable HPRT activity. Column 3 and 4 demonstrate that this HPRT activity was due to mycoplasma contamination. This contamination was detected by two independent methods: a. microbiologically (column 3) and b. by assaying adenosine phosphorylase in the culture medium according to Hatanaka *et al.* (8)(column 4).

TABLE II

Ultramicrochemical HPRT and PNP measurements in an uncontaminated and a mycoplasma contaminated Lesch-Nyhan fibroblast strain[a]

Cell strain	PNP-Ado in culture medium	HPRT activity[b] (10^{-13}moles/cell.hr)	PNP-Ad activity (10^{-13}moles/cell.hr)	PNP-Ado activity (10^{-16}moles/cell.hr)
GM 68	-	-0.01[c]	-0.5	-0.1
		0.01	2.3	0.1
		-0.04	1.0	0.1
		0.02	3.6	-0.1
		0.06	1.4	0.2
	mean (± s.d.)	0.01 (0.04)	1.6 (1.5)	0.0 (0.1)
GM 29	+	5.31	121.9	23.5
		7.89	155.3	43.1
		12.12	84.9	52.8
		8.17	90.2	37.9
		6.64	113.1	29.7
	mean (± s.d.)	8.03 (2.55)	113.1 (28.1)	37.4 (11.4)

[a] For each cell strain the 3 enzyme measurements were carried out in five fold; the cells were isolated from the same PFD.

[b] Mean value of normal controls 1.69 x 10^{-13}moles/cell.hr (range 1.38-2.42

[c] Blank is already subtracted. As the blank (average of five measurements) may be higher than individual enzyme determinations with these exceedingly low activities (due to methodological errors), individual negative values might be calculated.

Table II summarizes the results of ultramicrochemical measurements of HPRT, PNP-Ado and PNP-Ad in one uncontaminated (GM 68) and in one contaminated (GM 29) Lesch-Nyhan cell strain. The cells were again isolated from the same PFD's that we used in the study mentioned in table I. These quantitative data again demonstrate a complete correlation between appearance of HPRT activity (column 2), high activity of PNP-Ad (column 3), PNP-Ado (column 4) and contamination with mycoplasma (column 1). In addition, column 3 demonstrates that in the mycoplasma positive culture GM 29 each single cell has highly increased PNP-Ad activity.

Fig. 1 shows the results of 19 individual PNP-Ad determinations with one single cell each. The cells were isolated from a contaminated (GM 29) and an uncontaminated (GM 68) PFD. The blanks (indicated with 0) and the uncontaminated single cells (indicated with -) show only the substrate (adenine peak). All other positions, i.e. contaminated cells, display the product adenosine and its derivatives inosine and hypoxanthine. Fig. 2 shows measurements of PNP-Ado with 10 cells and of PNP-Ad with 1 cell. It is evident that in the PNP-Ado reaction the product (adenine) of 10 contaminated cells is hardly detectable, while in the PNP-Ad reaction the product (adenosine and its derivatives

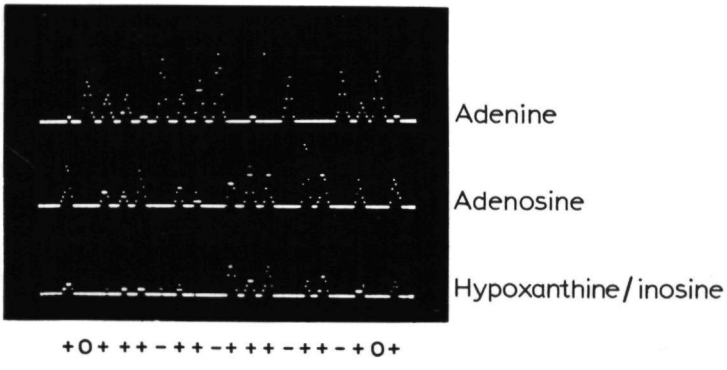

Fig. 1. Distribution of radioactivity on a chromatogram from 19 individual PNP-Ad measurements, read for 5 min per row with the Chromelec model 101. Upper row: adenine (substrate); middle row: adenosine (product); lower row: hypoxanthine and inosine (adenosine derived degradation products).
Positions indicated with 0: blanks.
Positions indicated with -: single cells from uncontaminated culture GM 68.
Positions indicated with +: single cells from mycoplasma contaminated culture GM 29.

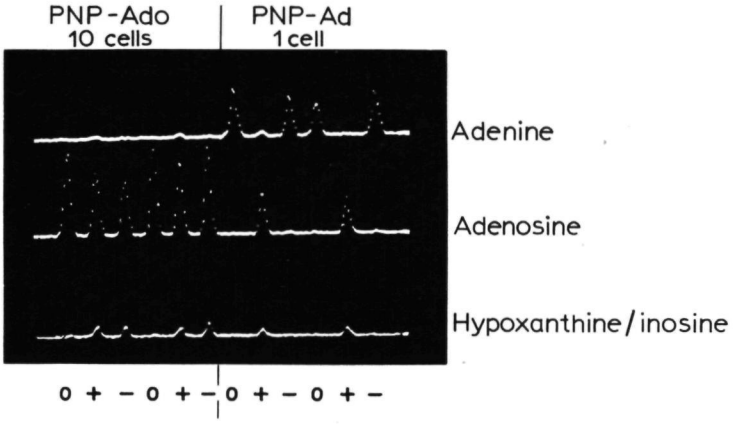

Fig. 2. Distribution of radioactivity on a chromatogram from six individual PNP-Ado (10 cells per determination) and six individual PNP-Ad (1 cell per dtermination) measurements, read for 10 min per row under the Chromelec model 101. Upper row: adenine (product of PNP-Ado reaction; substrate of PNP-Ad); middle row: adenosine (substrate of PNP-Ado; product of PNP-Ad); lower row: hypoxanthine and inosine (adenosine derived degradation products).
Positions indicated with 0: blanks.
Positions indicated with +: mycoplasma contaminated cells from GM 29.
Positions indicated with −: uncontaminated cells from GM 68.

inosine and hypoxanthine) of single contaminated cells is very clearly visible.

In fig. 1 and 2 the substrate peaks of blanks and uncontaminated cells do not have absolutely identical heights. This is caused by the fact that to speed up the procedure no attempts have been made to obtain completely quantitative data by exact micropipetting or precise centering of the rows of radioactive spots in the chromelec 101.

Discussion

In recent years it has become clear that cell cultures must continuously be monitored for mycoplasma contamination. This has become particularly obvious in the study of inborn errors which affect purine metabolism, like in the Lesch-Nyhan syndrome (9).

Several methods to detect mycoplasma are currently used, like DNA staining (21) or the microbiological isolation of the contaminants with special culture media. A bio-

chemical test, as proposed by Hatanaka *et al.* (8), would seem preferable (1), the more because it might be adapted to large scale screening procedures. As it seems likely that the major mycoplasma cell culture contaminants can be detected with an adenosine phosphorylase assay (8), we planned in a first phase to miniaturise the enzyme test and to adapt it later on to large scale screening. The miniaturisation is of special importance to the rapid prenatal diagnosis of genetic defects in early pregnancy (22).

From the results presented in this paper it is obvious that by choosing the PNP-Ad reaction it is possible to routinely detect mycoplasma contamination even in single cells (Table II).

The PNP-Ad values of strain GM 29 (Table II, column 3) are an underestimation of the real PNP-Ad activity, due to substrate exhaustion. In a separate experiment single cells from a contaminated culture were incubated for 7.5 min at 37°C to assay PNP-Ad and PNP-Ado. The PNP-Ad activity was 7×10^{-11} moles/cell.hr; the PNP-Ado activity was 2×10^{-15} moles/cell.hr, i.e. the nucleoside forming reaction is at least 35,000 times faster than the base forming reaction. For large scale screening of cell cultures it would be simpler to use the culture medium than the cells. The reason why we prefer to use the cells is that in case of low grade contaminations most mycoplasma particles are attached to the cell membranes and only when a certain level of saturation is reached mycoplasma, and consequently adenosine phosphorylase, will appear in the medium (23). In heavily contaminated cultures all single cells are adenosine phosphorylase positive. This has been demonstrated by testing 140 single cells isolated from several contaminated cultures.

The present ultramicromethod seems completely reliable and has already practically been used in the prenatal diagnosis of the Lesch-Nyhan syndrome (24; unpublished observations) and in experimental procedures (25). For large scale screening, however, it is too cumbersome. Therefore the PNP-Ad reaction is being adapted to a semimicromethod. A major role in such a mass screening will be played by a radiochromatogram reader.The results obtained with the one-dimensional Chromelec 101 (figs. 1 and 2) demonstrate the potentials of such an approach: no need to isolate the radioactive spots, considerable reduction of counting time, direct display. Centering problems with unidimensional readers make it imperative to develop a two-dimensional radiochromatogram reader.

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METABOLIC COOPERATION STUDIED BY A QUANTITATIVE ENZYME ASSAY OF SINGLE CELLS

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Summary

A new method making use of a radiochemical enzyme assay at the single cell level is presented to investigate metabolic cooperation, a widely studied form of cellular communication. In this case metabolic cooperation between normal human fibroblasts and fibroblasts derived from a patient deficient for the enzyme hypoxanthine-guanine phosphoribosyl transferase has been studied.

A mixture of an equal number of both cell types was cultured in close physical contact and after trypsinisation, replating and culturing the cells for several hours in a high dilution, quantitative enzyme measurements with individual cells isolated from the mixture were carried out. From the distribution curve of the enzyme activities of the individual cells the conclusion could be drawn that a macromolecule, either the enzyme itself or DNA or mRNA, coding for that enzyme, is transferred from normal to mutant cells.

Cultured skin fibroblasts from Lesch-Nyhan patients, deficient for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HG-PRT; EC 2.4.2.8), that have been grown in close contact with fibroblasts from normal individuals, are able to incorporate radioactive label added to the medium as ^{3}H-hypoxanthine into acid precipitable macromolecules, while in the absence of the HG-PRT positive cells no label is incorporated in the mutant cells (1). This phenomenon, called metabolic cooperation, has been studied by several groups, resulting essentially in two explanations: transfer from HG-PRT^{+} to HG-PRT^{-} cells of the enzyme product inosine monophosphate (IMP) (2,3) or transfer

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of macromolecules (HG-PRT itself, or DNA or mRNA containing the information for that enzyme) (4,5).

Indirect evidence for both hypotheses has been obtained by most of the groups employing autoradiographic demonstration of incorporation of radioactive label in the $HG\text{-}PRT^-$ cells and by turnover studies of the metabolic cooperation effect (2,4,5). A more straightforeward approach was reported by Oei and de Bruyn (3), who studied the transfer of labeled material from erythrocytes preloaded with ^{3}H-IMP to $HG\text{-}PRT^-$ lymphocytes or fibroblasts.

In the present communication the possibility of the transfer of enzyme or an informational macromolecule (DNA or mRNA) has been investigated by assaying HG-PRT activities of individual fibroblasts isolated from a mixture of $HG\text{-}PRT^+$ and $HG\text{-}PRT^-$ cells previously grown in close physical contact.

Materials and Methods

All chemicals used were obtained from Merck (analytical grade) except PRPP a grade, Calbiochem; bovine serum albumine, unlabeled hypoxanthine, inosine, IMP, Sigma and ^{14}C-labeled hypoxanthine, Radiochemical Centre Ltd. Amersham. The experimental procedure was as follows: $HG\text{-}PRT^+$ and $HG\text{-}PRT^-$ cultures of human fibroblasts were seeded in a 1:1 ratio, grown for one day to confluency and kept in confluency for three further days in HAM F 10 medium containing 15% fetal calf serum and 100 I.U./ml penicillin and 100 μg/ml streptomycin. Subsequently, the cells were trypsinised, washed three times, resuspended in culture medium and seeded into a Plastic Film Dish (PFD; 6) in such a way that there was no more contact between the cells, i.e. $1x10^5$ cells per 20 cm^2 of culture surface. Eight hours later the cells were washed and lyophilized *in situ*. About 100 individual cells were cut out from the plastic film bottom of the PFD under a stereomicroscope and tested for HG-PRT activity. As controls the $HG\text{-}PRT^+$ and $HG\text{-}PRT^-$ cultures were grown in separate PFD's and treated the same way. The techniques to measure enzyme activities at the single cell level (7,8) and their adaptation to radiochemical assays of purine phosphoribosyl transferase activities (9,10) have been developed and described previously.

The final assay mixture contained 0.17 M Tris-HCl buffer (pH 7.4), 17 mM $MgCl_2$, 1.7 mM PRPP, 0.13 mM ^{14}C-hypoxanthine (spec.act. 52 mCi/mmole), 1.6 mg/ml streptomycin and 0.5°/oo BSA. The incubation volume was 0.3 μl. Except for the blanks, in each reaction mixture a plastic film leaflet, carrying the single cell to be assayed, was included.

After incubation for six to seven hours at 37°C in Parafilm Micro Cuvettes (PMC; 6) the reaction was stopped by pressing the PMC's onto Whatmann 3MM paper strips. Separation of substrate and product was carried out with a 0.5 N ammonia, 0.05 N EDTA solution as eluent. Quantification of radioactivity was performed as described previously (10).

Results and Discussion

The distribution of the HG-PRT activities in both the normal positive and the mutant

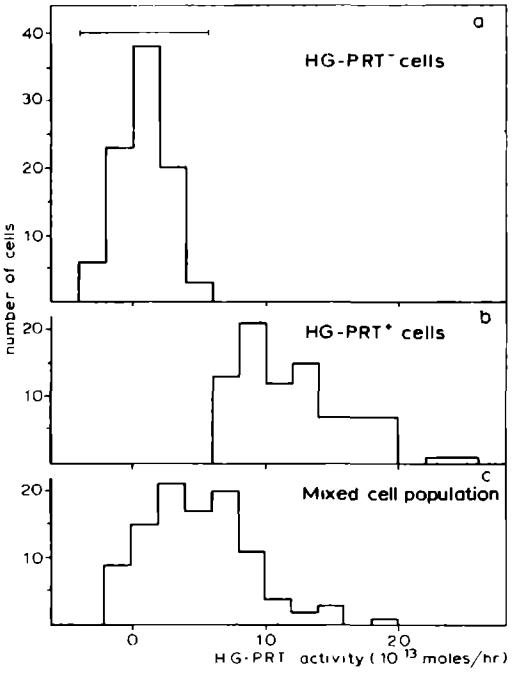

Figure 1.

a. Frequency distribution of enzyme activities of individual HG-PRT$^-$ fibroblasts (n=90). In addition, the range of the blank measurements is indicated (bar on top).
b. Frequency distribution of enzyme activities of individual HG-PRT$^+$ fibroblasts (n=84).
c. Frequency distribution of enzyme activities of individual fibroblasts (n=103), isolated from a mixture of HG-PRT$^+$ and HG-PRT$^-$ cells grown in close physical contact in a 1:1 ratio.

negative control fibroblasts are shown in figs.1a. and 1b. The mean value of the HG-PRT activities in the individual deficient control cells was 0.07×10^{-13}moles/hour. The corresponding value for the normal control cells was 1.22×10^{-13}moles/hour. The mean value of the blank measurements (n=19) was defined as 0×10^{-13}moles/hour. The range was between 0.40 and $+0.56 \times 10^{-13}$moles/hour as is indicated in fig.1a. (bar on top). AS can be seen the activity range of the HG-PRT deficient cells is comparable.

If no transfer of enzyme- or informational molecules from HG-PRT$^+$ to HG-PRT$^-$ cells would occur during cellular contact in a mixed cell population, the enzyme activity distribution of the individual cells would have been a combination of figs. 1a. and 1b.

As can be seen from fig. 1c. the observed distribution in the cell mixture was fundamentally different. Instead of two peaks to be expected for both cell types in the mixture, only one peak with intermediate HG-PRT activities was found (average activity 0.51×10^{-13} moles/hour).

These findings indicate that HG-PRT$^+$ and HG-PRT$^-$ fibroblasts grown to confluency constitute a communicating system, i.e. during cellular contact either HG-PRT or an informational macromolecule, coding for the enzyme, is transferred from cell to cell. The experiment has been repeated several times with different combinations of the HG-PRT$^+$ and HG-PRT$^-$ human cell cultures, always leading to the same conclusion.

Increase of enzyme activity in the HG-PRT$^-$ cells and decrease of enzyme activity in the HG-PRT$^+$ cells, as suggested by the data in fig.1, could be demonstrated more quantitatively if the distribution of the enzyme activities of the individual cells would be less effected by methodological errors. This could be achieved by prolonging the liquid scintillation counting time and by improving the micro-pipetting accuracy.

The method used here (i.e. the quantitative HG-PRT measurement in individual normal and mutant cells after cellular contact) differs from the methods used by other groups with respect to the following points:

1. In this study both the HG-PRT$^+$ and HG-PRT$^-$ cells had been interacting under relatively physiological conditions, in contrast to for example the experiments of Ashkenazy and Gartler (5), who studied metabolic cooperation by adding a lysate of HG-PRT$^+$ fibroblasts to a culture of HG-PRT$^-$ cells.
2. The method used here offers an opportunity to specifically study a possible transfer of enzyme- or informational molecules, since the HG-PRT activity is radiochemically measured and the radioactive substrate is added to the individual cells after they have been isolated; a possible transfer of metabolites during cell to cell contact cannot interfere with the enzyme assay.

A complementary approach to specifically study transfer of metabolites from cell to cell was reported previously by our group (3). The methods used by other groups which are based on autoradiographic demonstration of incorporation of labeled hypoxanthine can, on the other hand, not discriminate between these two possibilities.

Whereas previously the transfer of metabolite from cell to cell was demonstrated (2, 3,11), the present study provides evidence for an additional mechanism of metabolic co-

operation between HG-PRT$^+$ and HG-PRT$^-$ cells. Further studies should elucidate if both, none, or only one of these mechanisms have any physiological significance.

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EVIDENCE FOR THE EXISTENCE OF DIFFERENT TYPES OF METABOLIC COOPERATION

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INTRODUCTION

Intercellular communication is supposed to be involved in a number of biological processes. For example, control of embryonic differentiation seems to require some form of close cellular interaction (1); the same has been suggested with respect to cell division and immune response (2,3).

One particular form of cell communication which has received relatively much attention during the past few years is metabolic cooperation. As a result of this phenomenon the mutant phenotype of certain enzyme deficient cells is corrected by contact with normal cells (4,5). Such a correction has been observed with hypoxanthine-guanine phosphoribosyl transferase deficient ($HG\text{-}PRT^-$) cells in culture. Normal $HG\text{-}PRT^+$ fibroblasts incorporate radioactive hypoxanthine or guanine into intracellular nucleotides demonstrable at the cellular level by autoradiography. Skin fibroblasts from patients with severe HG-PRT deficiency show a marked reduction in incorporation of these nucleotides under similar conditions. However, $HG\text{-}PRT^-$ fibroblasts grown in close contact with normal fibroblasts become labeled (6,7,8).

In most studies published thus far, intercellular exchange has been studied with cultured skin fibroblasts. Normal and $HG\text{-}PRT^-$ cells were cocultured in a ^{3}H-hypoxanthine containing medium and after incubation the cells were monitored for radioactivity by means of autoradiography.

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In the present study metabolic cooperation has been analysed in two different ways: one method made use of normal donor cells (lymphocytes or erythrocytes) which were preincubated in ^{3}H-hypoxanthine containing medium (9). These cells were brought into contact with HG-PRT$^-$ recipient cells (fibroblasts or lymphocytes) and eventual transfer of label was studied by autoradiography. The second way was to use a recently developed HG-PRT activity measurement (10,11) of single fibroblasts isolated from a 1:1 mixture of normal and HG-PRT$^-$ cells which had previously been growing in close physical contact. In this way eventual appearance of HG-PRT activity in the HG-PRT$^-$ fibroblasts could be monitored.

MATERIALS AND METHODS

Autoradiography. These experiments were carried out with HG-PRT$^-$ fibroblasts and HG-PRT$^-$ lymphocytes as recipient cells. Fibroblasts were cultured in HAM F-10 medium containing antibiotics (penicillin 100 U/ml; streptomycin 100 γ/ml) and 15% fetal calf serum. Lymphocytes were isolated from fresh heparinised blood according to Roos and Loos (12). As donor cells normal lymphocytes and normal erythrocytes were used. Erythrocytes, retained after removal of plasma and buffy coat from heparinised blood, were washed three times with isotonic phosphate buffer (pH 7.4) before being used.

The normal donor cells were preincubated in a medium which contained per ml: 0.3 ml isotonic Na, K-phosphate buffer (pH 7.4), 0.45 ml of 0.9% NaCl, 0.05 ml of 2.35% $MgCl_2$ and 0.2 ml of 1% glucose; 8-^{3}H-Hypoxanthine (Radiochemical Centre, Amersham; spec.act.500 mCi/mmol) was included in a concentration of 0.02 mM. Lymphocytes were preincubated for 180 min. and erythrocytes for 60 min. at 37^{o}C. Subsequently the cells were washed out untill the washing fluid contained no more radioactivity (normally after 2-3 washings). The preincubated and washed lymphocytes or erythrocytes were mixed with untreated HG-PRT$^-$ lymphocytes from a patient with the Lesch-Nyhan syndrome and spun down to assure cellular contact. In addition, preincubated control erythrocytes were allowed to sediment on top of HG-PRT$^-$ fibroblasts grown on coverslips. After incubation for 16 to 18 hrs, erythrocytes were removed by osmotic shock and the remaining cells (fibroblasts or lymphocytes) were submitted to autoradiography (13).

In a number of experiments the incorporation of label added to

the medium as ^{3}H-IMP (2 µM) was studied in the presence of crude particulate fractions from normal and HG-PRT deficient erythrocytes and fibroblasts. These fractions were obtained after disruption of the cells by repeated freezing-thawing and centrifugation (1,000 g; 20 min) of the lysate. After several washings the pellets were suspended (10 γ/ml) in culture medium, containing ^{3}H-IMP, and allowed to sediment on top of HG-PRT deficient fibroblasts growing in monolayer. Following incubation for 16-18 hrs at 37°C autoradiography was performed as described above.

Measurement of HG-PRT in single cells. The method to assay activities at the single cell level has originally been developed for fluorogenic substrates (14) and adapted recently for radiochemical assays (10,11). Briefly, a 1:1 mixture of normal and HG-PRT$^-$ fibroblasts is grown in confluency during 3 days in a Plastic Film Dish (PFD; 14). After trypsinisation, the cell suspension is diluted and replated; 8 hours later the cell culture is lyophilised and some 100 individual cells are cut out, free-hand under a stereomicroscope, from the bottom of the PFD. Enzyme activities are measured in Parafilm Micro Cuvettes (PMC's), small disposable incubation vessels, containing 0.3 µl of incubation medium and a plastic film leaflet carrying the single fibroblast to be tested. Substrate and product are separated by paper chromatography and enzyme activity per cell is calculated after quantification of radioactivity in a liquid scintillation counter.

RESULTS

Table I shows the autoradiographic experiments with the experimental mixtures. Intercellular exchange of label has taken place in the combinations lymphocyte ⟶ lymphocyte; erythrocyte ⟶ lymphocyte; and erythrocyte ⟶ fibroblast. Most label in the recipient (HG-PRT$^-$) cells was noticed in the nuclear regions.

High voltage electrophoresis and paper chromatography has shown that the radioactivity in the donor erythrocytes immediately after preincubation was predominantly present as ^{3}H-IMP (>95%). Therefore, the material transferred to the HG-PRT$^-$ lymphocytes or fibroblasts might be IMP or a derivative which could be incorporated by the recipient cells. ^{3}H-IMP added to the medium did not produce labeling of the HG-PRT$^-$ cells (table I). However, addition to the medium of crude particulate fractions from fibroblasts or erythrocytes (both HG-PRT$^+$ or HG-PRT$^-$), along with ^{3}H-IMP, induced incor-

TABLE I

Metabolic cooperation in experimental cell mixtures.

a. intact donor and recipient cells

preincubated donor cells:	recipient cells:	appearance of label in recipient cells:
normal lymphocytes	HG-PRT$^-$ lymphocytes	yes
normal erythrocytes	HG-PRT$^-$ fibroblasts	yes
normal erythrocytes	HG-PRT$^-$ lymphocytes	yes

b. only intact recipient cells

added to the medium:	recipient cells:	
^{3}H-IMP (20 µM)	HG-PRT$^-$ fibroblasts	no
^{3}H-IMP (20 µM)	HG-PRT$^-$ lymphocytes	no
^{3}H-IMP + crude particulate fraction (normal erythrocytes)	HG-PRT$^-$ fibroblasts	yes
^{3}H-IMP + crude particulate fraction (normal fibroblasts)	HG-PRT$^-$ fibroblasts	yes
^{3}H-IMP + crude particulate fraction (HG-PRT$^-$ erythrocytes)	HG-PRT$^-$ fibroblasts	yes

poration in HG-PRT$^-$ fibroblasts (figure 1). Heavy labeling was seen in both nucleus and cytoplasm.

The frequency distribution of HG-PRT activities in individual control fibroblasts and fibroblasts from a patient with the Lesch-Nyhan syndrome did not show overlap (fig.2). The activities of cells from a 1:1 mixture of normal and mutant fibroblasts which had been growing in close contact showed a completely different distribution, suggesting a communicating cell system (fig.2). The appearance of HG-PRT activity in HG-PRT$^-$ cells is indicative of the transfer of the enzyme itself, or an informational molecule, or a regulatory molecule.

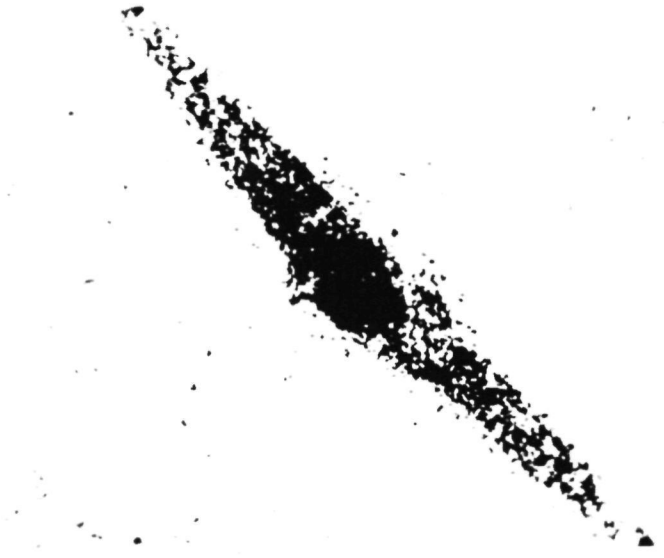

Figure 1.
TCA precipitable label in a HG-PRT deficient fibroblast after incubation with ^{3}H-IMP and a crude particulate fraction from normal cultured fibroblasts.

DISCUSSION

The evidence in favour of the transfer of enzyme product from normal to HG-PRT$^-$ cells as a basis for metabolic cooperation is well documented (7,8,9,15), whereas far less evidence in favour of the possible transfer of other compounds have been published.

From present and previous (9) data it is evident that metabolic cooperation can occur not only between cultured normal and HG-PRT deficient fibroblasts, but also between normal and HG-PRT deficient lymphocytes and between normal red blood cells and HG-PRT deficient lymphocytes or fibroblasts. A basis for this phenotypic correction of mutant cells might indeed be the transfer of IMP or a derivative.

Ashkenazi and Gartler (16) added lysate from normal fibroblasts

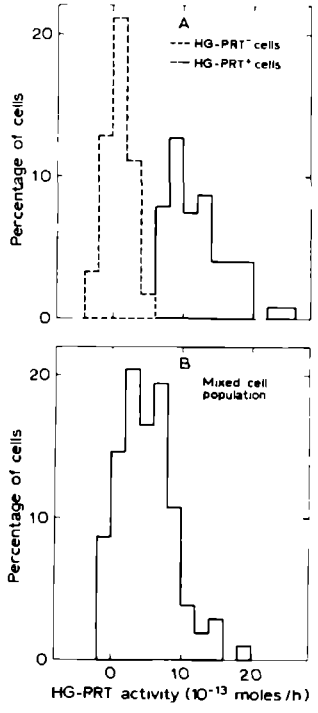

Figure 2.
Frequency distribution of HG-PRT activities in individual fibroblasts.
A: enzyme activities from non-interacting control and HG-PRT deficient cultures. About 100 cells of each culture were assayed.
B: enzyme activities from a mixed culture in which normal and HG-PRT deficient cells have been cocultured in a 1:1 ratio. About 100 individual cells were assayed.

to a culture of HG-PRT$^-$ fibroblasts and this caused incorporation of label originally added to the medium as ^{3}H-hypoxanthine. Autoradiographic examination revealed most label to be cytoplasmic, in contrast to the nuclear labeling found by other investigators who used intact donor cells. It has been inferred that metabolic cooperation is efficient when radioactive labeling is primarily located in the nucleus and inefficient if the label is cytoplasmic

(15). When incubating HG-PRT$^-$ fibroblasts with crude particulate fractions and ^{3}H-IMP, we observed heavy labeling of both nucleus and cytoplasm. Therefore, on the basis of the findings obtained with autoradiography, there might be considerable heterogeneity with respect to the mechanisms and the compounds involved in intercellular communication.

The appearance of HG-PRT activity in HG-PRT$^-$ fibroblasts after coculturing with normal fibroblasts, as judged from single cell enzyme measurements, is the first direct evidence of exchange of material leading to enzyme activity in mutant cells. Eventual transfer of (unlabeled) enzyme products in these experiments will not interfere with the enzyme assay, since the HG-PRT activity is determined with radioactive substrate after the cells have been in contact (17), and isolated from the population. A possible source of error could be a contamination of HG-PRT$^-$ cells with membrane associated HG-PRT from normal cells which might be released when the confluent is trypsinised. However, the quantitative significance of such a contamination as a result of a routine cell culturing procedure seems questionable.

The presence of HG-PRT activity in part of the HG-PRT$^-$ fibroblasts was established eight hours after replating of the cells which had been grown to confluency previously. Apparently this type of exchange between intact cells differs from the mechanism observed when studying the transfer of a low molecular weight metabolite: in that case HG-PRT$^-$ cells promptly reverted to the mutant phenotype when separated from normal donor cells (8,18).

It has been suggested that the ability of cells to communicate might be correlated with the presence of specific structures in the cell membrane. These structures, called low resistance- or gap junctions, have been demonstrated in cells which were ionically and metabolically coupled (19). On the other hand it has been reported that communication may also take place among cells that apparently lack gap junctions: after prolonged interaction "low efficiency cooperation" was observed (15). It could be possible that exchange took place via some undetected gap junctions, but an interesting alternative would be that it might represent a more universal form of cell communication, i.e. the exchange of molecules located on or in the membranes of the apposed cells. In this context it might be relevant to look for nucleotide metabolising enzyme systems associated with the cellular membrane. An indication

in this direction is the formation of labeled extracellular IMP or GMP when intact erythrocytes are incubated in a medium containing radioactive hypoxanthine or guanine and phosphoribosylpyrophosphate, the co-substrate of the HG-PRT reaction (20,21).

The concept has evolved that intercellular communication constitutes an important mechanism for control of metabolic activity and regulation of growth, differantiation and embryonic development. It must be recognised, however, that without being able to discriminate between the various mechanisms of cell communication and the various molecules transferred, evidence for its role in vivo is largely inductive.

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QUANTITATIVE STUDIES ON TWO TYPES OF METABOLIC COOPERATION BETWEEN NORMAL ERYTHROCYTES AND HPRT DEFICIENT FIBROBLASTS

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Abstract

Two previously demonstrated types of metabolic cooperation were investigated using normal erythrocytes as donor and hypoxanthine phosphoribosyl transferase (HPRT) deficient fibroblasts as recipient cells. When erythrocytes, preloaded with ^{3}H labeled IMP, were brought in contact with HPRT deficient fibroblasts, labeled material was transferred to the fibroblasts, where it was incorporated into acid precipitable material. The amount of incorporation depended on the amount of preloaded erythrocytes added. It was shown that red cell lysate is about an equally efficient donor as intact erythrocytes. Another form of metabolic cooperation, transfer of HPRT or informational molecules leading to HPRT activity, was proven not to occur under the same conditions, neither with intact, nor with lysed erythrocytes as donor. Apparently only one of the two forms of metabolic cooperation studied exists in the erythrocyte-fibroblast system.

Metabolic cooperation is a form of cell communication by which the mutant phenotype of certain enzyme deficient cells is corrected by contact with normal cells (Subak-Sharpe et al., '66, '69). Metabolic cooperation between normal and hypoxanthine phosphoribosyl transferase (HPRT; EC 2.4.2.8) deficient cells has been explained in two ways: 1. transfer of the enzyme product IMP or a related compound (Cox et al., '70, '72), and 2. transfer of macromolecules (such as HPRT, informational molecules like DNA and mRNA or an HPRT activating protein) (Fujimoto and Seegmiller, '70; Ashkenazy and Gartler, '71). Transfer of IMP or a related metabolite between communication-competent fibroblasts is believed to go through gap junctions (Azarnia et al., '72; Gilula et al.,

'72). Autoradiographic detection of this type of metabolic cooperation after the cells have been incubated with radioactively labeled hypoxanthine for only two hours (Cox et al., '70, '72; Azarnia et al., '72; Gilula et al., '72). In a system with $HPRT^+$ and $HPRT^-$ fibroblasts also transfer of compounds leading to HPRT activity could be proven (Uitendaal et al., '76). Therefore it is concluded that between fibroblasts both metabolites and compounds leading to HPRT activity can be exchanged.

Mouse L cells, that lack gap junctions, also exhibit metabolic cooperation, when long incubation times (18 hours) with hypoxanthine are used (Cox et al., '74). Similarly, after long incubation times, transfer of metabolites has been proven to occur from red blood cells, loaded with radioactive IMP, to $HPRT^-$ fibroblasts (Oei and de Bruyn, '74). Red blood cells are probably not capable of forming gap junctions and this type of metabolic cooperation might very well differ from the metabolite transfer observed between fibroblasts (Azarnia et al., '72; Gilula et al., '72).

Both transfer of metabolites and of compounds leading to HPRT activity were studied in a system of red blood cells or a lysate of those cells as donor and $HPRT^-$ fibroblasts as the recipient cells. A dosage effect of IMP-loaded donor cells in metabolic cooperation is also studied.

Materials and Methods

All chemicals were obtained from Merck (analytical grade) except PRPP, A grade, Calbiochem; ^3H -hypoxanthine (2 Ci/mmol) and [8-^{14}C]-hypoxanthine (59 mCi/mmol), Radiochemical Centre, Amersham. Normal and $HPRT^-$ cells were skin derived fibroblasts from respectively a healthy individual and a patient with the Lesch-Nyhan syndrome and were cultured in medium 199 containing antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml) and 15% fetal calf serum in 25 cm^2 Falcon bottles.

Erythrocytes from a normal individual were loaded with ^3H -IMP as described before (Oei and de Bruyn, '74). ^3H -IMP loaded as well as untreated erythrocytes were lysed by sonicating the washed, packed cells for three times three seconds at maximal power with a Branson model MS2T with microtip at 0°C. Electrophoretic analysis of the labeled lysate showed that more than 90% of the label was in the form of IMP. ^3H -IMP was purified from the lysate by paper electrophoresis.

Transfer of enzyme product was investigated by adding 60 μl of the packed, ^3H -IMP loaded, donor erythrocytes (or an equivalent amount of lysate) to the medium of in log-phase growing recipient $HPRT^-$ fibroblasts. After an incubation of 16 hours at 37°C the

medium with the erythrocytes or lysate was removed and the cells were washed with medium 199 until the washing fluid contained no more radioactivity (normally after 6 washings). Subsequently, 2 ml of a 5% trichloroacetic acid solution were added to the fibroblasts at $4^{\circ}C$ and the precipitate was washed two times. The precipitate was dissolved in 0.5 ml 0.1 N NaOH and aliquots were taken for counting in a Packard 2450 liquid scintillation counter and for protein determination.

Transfer of HPRT or informational macromolecules was investigated by adding 60 μl washed, packed donor erythrocytes (or an equivalent amount of lysate) to the medium of in log-phase growing recipient $HPRT^-$ fibroblasts. After an incubation of 16 hours at $37^{\circ}C$, the medium was removed and the fibroblasts were washed six times with medium 199. The fibroblasts, still in the culturing bottle, were lyophilized overnight and afterwards dissolved in 0.75 ml distilled water. Aliquots were taken for determination of HPRT activity and protein content.

HPRT activity was measured by incubating the lyophilized cells in a 30 μl mixture containing 0.17 M Tris-HCl buffer (pH 7.4), 17 mM $MgCl_2$, 6.7 mM PRPP and 0.267 mM [8-^{14}C]-hypoxanthine for 2 hours at $37^{\circ}C$. Separation of substrate and product was carried out with reference compounds by descending chromatography on Whatmann 3 MM paper with 0.5 N ammonia, 0.05 N EDTA as eluent. The spots, visible under UV light, were cut out and counted in a liquid scintillation counter. The radioactive counts on the IMP and inosine spots were added to determine total HPRT product formation. One unit (U) of HPRT is that amount of enzyme that converts one mmole hypoxanthine per minute. Protein was determined according to Lowry et al. ('51).

Results and Discussion

Addition of ^{3}H -IMP to the medium of $HPRT^-$ fibroblasts leads to a certain incorporation of label in acid precipitable material (Table 1). When erythrocytes containing the same amount of ^{3}H -IMP are incubated with $HPRT^-$ fibroblasts, an increase of the incorporation in acid precipitable material is observed (Table 1). When less IMP-loaded erythrocytes are brought onto the recipient fibroblasts, the stimulation of ^{3}H incorporation also decreases (Table 1). Also, when lysates of IMP-loaded erythrocytes are used as donor, the incorporation in the fibroblasts appears to be dependent on the amount of lysate added (Table 1). As can be seen in table 1, the increase of ^{3}H incorporation was of the same order of magnitude with either intact or lysed IMP-containing erythrocytes as donors. $HPRT^-$ fibroblasts incorporated only a fraction (1.2%) of label-

TABLE I

Incorporation of radioactivity into acid precipitable material by HPRT⁻ fibroblasts after an incubation of 16 hours at 37°C

fibroblasts	packed erythrocytes added to the medium		labeled compounds in medium (10^6 dpm)		radioactivity incorporated (10^3 dpm/mg protein)
	intact	sonicated			
HPRT⁻	-	-	25.0	IMP[1]	9.0
HPRT⁻	60 µl	-	25.0	IMP	88.0
HPRT⁻	15 µl	-	6.25	IMP	31.8
HPRT⁻	-	60 µl	25.0	IMP	100.4
HPRT⁻	-	15 µl	6.25	IMP	25.9
HPRT⁻	-	-	25.0	Hx[2]	10.9
normal	-	-	25.0	Hx	918.9

1) Equivalent to 5.7 nmoles IMP.

2) Hx = hypoxanthine. Equivalent to 5.7 nmoles.

ed hypoxanthine into acid precipitable material as compared to normal fibroblasts (Table 1).

Transfer of HPRT did not occur after contact with unloaded erythrocytes, neither intact, nor lysed (Table 2); no increase of HPRT activity was found in the HPRT⁻ fibroblasts. Also addition of more erythrocytes did not lead to HPRT activity in the mutant fibroblasts. Normal fibroblasts did not show a higher HPRT activity either, when incubated with HPRT containing erythrocytes.

The background incorporation in the mutant fibroblasts after 16 hours incubation with ^{3}H -IMP (Table 1) can be explained as follows. Three hours after the IMP is added, it is degraded to ^{3}H -hypoxanthine (data not shown). The residual HPRT activity of the mutant cells (Table 2) enables them to incorporate hypoxanthine into acid precipitable material with about a 1% efficiency as compared to control cells (Table 1).

There is evidence for at least two forms of metabolic cooperation; transfer of meta-

TABLE 2

HPRT activity of HPRT⁻ fibroblasts after incubation of 16 hours at 37°C with intact and lysed erythrocytes

fibroblasts	packed erythrocytes added to the medium		HPRT added (mU)	HPRT activity in fibroblasts (mU/mg protein)[1]
	intact	sonicated		
HPRT⁻	-	-	-	0.09
HPRT⁻	60 µl	-	19.73	0.08
HPRT⁻	180 µl	-	59.19	0.08
HPRT⁻	-	60 µl	19.73	0.10
normal	-	-	-	3.21
normal	60 µl	-	19.73	3.12

[1] Normal value: mean 3.23 mU/mg; range 2.86 - 3.70 mU/mg protein.

bolites and transfer of material leading to HPRT activity in the mutant cells (de Bruyn et al., '77). Transfer of IMP or a derivative occurs in many donor cell-recipient cell combinations (Cox et al., '70; Oei and de Bruyn, '74), including normal erythrocytes with HPRT⁻ fibroblasts (Oei and de Bruyn, '74)(Table 1), although in the last case longer incubation times are required. In addition, IMP can be transferred to HPRT⁻ fibroblasts not only from erythrocytes, but also from the medium, provided that there is also hemolysate (Table 1) or a crude membrane preparation present (Oei and de Bruyn, '74). Whether the dosage effect, shown in table 1, depends on the amount of biochemically active material (possibly membrane components), is still under investigation.

Erythrocytes are believed to be incapable of gap junction formation and radioactivity incorporation into the recipient cells is demonstrable only after long incubation times, in contrast to incorporation after IMP passage through gap junctions in the fibroblast-fibroblast system (Cox et al., '70, '72; Azarnia et al., '72; Gilula et al., '72). Therefore, transport of IMP from erythrocytes to fibroblasts has to be explained by an other mechanism. Although pinocytosis by the fibroblasts of loaded erythrocytes or cell fragments with unspecifically bound ^{3}H -IMP cannot be definitively excluded,

we consider this alternative improbable: 1. Pinocytosis is not demonstrable in the parallel experiment (Table 2), neither of HPRT in intact erythrocytes, nor of HPRT in erythrocyte lysate, although HPRT is partly membrane associated (de Bruyn and Oei, '77). 2. Labeled IMP, concentrated in the intact erythrocytes, and ^{3}H -IMP, diluted in the culturing medium and maybe partly bound to membrane fragments of the lysed erythrocytes are incorporated equally efficient (Table 1). 3. Pinocytosis implicates contact with the fibroblast membrane, that is known to contain 5'-nucleotidase, and uptake into lysosomes with lysosomal phosphatases. Nevertheless, the nucleotide reaches the cytoplasma without being degraded.

The mechanism, by which IMP is transferred, might be related to that which is responsable for metabolic cooperation in L cells (Azarnia et al., '72; Gilula et al., '72), although as yet that mechanism is also not understood.

The second form of metabolic cooperation, transfer of information leading to HPRT activity, has been demonstrated until now exclusively in a system where both donor and recipient cells were fibroblasts (Uitendaal et al., '76). Here this form of metabolic cooperation is shown to be absent between normal erythrocytes and HPRT^{-} fibroblasts (Table 2) under conditions that permit the first form of intercellular communication (Table 1). The absolute amount of HPRT added to the fibroblasts in this experiment (up to 59.19 mU), however, was in principle sufficient; when a 1:1 mixture of normal and HPRT^{-} fibroblasts are cultured together, the total amount of HPRT in one bottle is only 0.86 mU (as calculated on the basis of HPRT activity in the normal fibroblasts in Table 2) and then the mutant cells aquire HPRT activity (Uitendaal et al., '76). Apparently erythrocytes are not capable of making the kind of contact needed for transfer of macromolecules.

The underlying mechanisms of these two forms of metabolic cooperation are not known. Therefore, no explanation can be given for the presence of one and absence of the other form in the erythrocyte-fibroblast system.

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PAPER 10

PHENOTYPIC INTERACTION: METABOLIC COOPERATION BETWEEN CELLS IN CULTURE

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CONTENTS

1.1 *Phenotypic Interaction*

The phenotype of cells in vitro can be influenced in different ways. After e.g. fusion of two mutant cells, the two genomes gathered into one cell can in many cases correct for each others mutation. Consequently, the resultant hybrid cell will be phenotypically normal, both in the case of intergenic (83) and interallelic (65) complementation. The phenotypic correction in hybrids between cells of different species can also be the result of the influence that one genome has on the expression of genes on the other (98,9). Another form of phenotypic correction is caused by uptake of isolated chromosomes from normal donor cells into mutant recipient cells. Genes located on these chromosomes can sometimes come to expression either after loss of the main part (59) or with preservation (101) of the chromosomes. The phenotype of mutated cells can also be corrected by addition of chemical compounds to the medium as is the case when hypoxanthine phosphoribosyl transferase (HPRT) deficient lymphocytes are reverted to normal by the action of phytohemagglutinin (25).

Still another way by which the phenotype of cells in culture can be altered is exchange of compounds between cells in a mixed culture of different cell strains. Intercellular communication is required for a variety of biological processes in vivo and in vitro, including contact inhibition of cell locomotion (1,2) and probably cell division (93,88), control of embryonic differentiation (29), induction of immune response (53), stimulation of lymphocytes by phytohemagglutinin and pokeweed mitogen (72) and agglutination of transformed cells by concanavalin A (85). This review will, however, confine itself to one particular form of intercellular communication, i.e. metabolic cooperation, originally described by Subak-Sharpe, Bürk and Pitts (91,92). This phenotypic correction of mutant by contact with normal cells can have disturbing effects upon several experimental procedures in vitro. In dense cultures of fibroblasts (intercellular contact) from heterozygotes for the Lesch-Nyhan syndrome (severe HPRT deficiency; 56,80), all cells are phenotypically normal (61,79.35), although according to the Lyon hypothesis (58) heterozygotes for this sex linked inbor error of metabolism are mosaics with respect to HPRT activity, as is clear in more diluted cultures (24,62, 35). And not only in heterozygote detection for X-linked enzyme deficiencies metabolic cooperation can be a complicating factor. Also, mutation frequency studies based on the appearance of HPRT deficient cells in a culture of normal cells can lead to an under-

estimation of the mutation frequency, because mutated cells in close contact with normal cells behave as normal cells due to metabolic cooperation (96).

On the other hand, metabolic cooperation offers some advantages to scientific research, because it is a relatively easy manipulated and analysable model for cell communication (21).

About the role in vivo of metabolic cooperation very little is known. Phenotypic correction of HPRT deficient cells by HPRT positive cells in vivo in heterozygotes for the Lesch-Nyhan syndrome has been reported (34), but no other example of metabolic cooperation in vivo involving any other mutated enzyme has been studied. Consequently, hypotheses about the physiological significance of metabolic cooperation are purely speculative.

Metabolic cooperation has been defined as the process whereby the metabolism of cells in contact is modified by exchange of material (91,92). This is a somewhat limited definition, since it excludes all forms of intercellular communication with metabolic implications that do not require cell contact. The correction of e.g. the mutant phenotypes in a mixed culture of fibroblasts from both Hurler and Hunter patients (two diseases in the group of mucopolysacharidoses) is not a form of metabolic cooperation, simply because these cells are mutated in different lysosomal enzymes and correction occurs by means of exchange of the non-mutated enzymes through the medium, i.e. the communicating cells are not necessarily in contact (66). This form of intercellular communication, that occurs with many lysosomal enzymes (66,78), will not extensively be discussed here as a form of metabolic cooperation. This review will make use of the definition by Subak-Sharpe. Nevertheless, some forms of cellular communication not needing cellular contact will be mentioned, because they are relevant for the understanding of metabolic cooperation and because the need for contact is not yet being investigated or still debatable.

1.2 *Metabolic Cooperation*

The definition for metabolic cooperation that is discussed in the previous paragraph does not explicitly mention the nature of the compound transferred. In principle transfer of five types of substances can lead to metabolic cooperation (92; see also chapter 4). In addition, the definition does not lay a restriction on the mechanisms by which the transfer is effectuated (see chapter 5). Therefore it is very well possible that there are several forms of metabolic cooperation existing together. This opinion

is strengthened by the finding that some cells are classified as either capable or incapable of metabolic cooperation depending on the method applied (see chapter 2) or the choise of recipient cells (see chapter 3).

While most of the studies published try to find one explanation for all cases of metabolic cooperation, in this review the literature is discussed in the light of the theory that there are different forms of metabolic cooperation.

In chapter 2 the influence of the method applied on the type of metabolic cooperation studied is discussed and chapter 3 deals with the influence of the choice of cell system. Chapter 4 talks about the different substances that can be exchanged between cells and in chapter 5 the possible mechanisms for this are discussed. Finally it is concluded in chapter 6 that there are at least two forms of metabolic cooperation. A tentative classification of the different forms studied thus far is given and the possible relations between those forms are mentioned.

2 *METHODS*

2.1 *Incorporation of Radioactive Nucleotides*

The first and most widely applied technique to study metabolic cooperation is autoradiography (91,24,92,18,35,96,62,67,32,74). Normal fibroblasts incorporate label into acid precipitable material (mainly nucleic acids) when radioactive hypoxanthine is added to the medium. This can be demonstrated autoradiographically. HPRT deficient fibroblasts have a markedly reduced incorporation, because they lack the possibility to convert hypoxanthine into its corresponding nucleotide IMP, a precursor for nucleic acid synthesis. These mutant cells do, however, show incorporation when grown in close contact with normal cells. As a consequence, an autoradiogram of a mixture of HPRT positive and HPRT negative cells show cells without grains (HPRT negative cells), heavily labeled cells (HPRT positive cells) and, whenever metabolic cooperation occurs, less heavily labeled cells (recipient HPRT deficient cells) in contact with heavily labeled donor cells. Counting the number of grains per cell can render this assay more or less quantitative (18,73). The same technique is also applicable for metabolic cooperation between normal and adenine phosphoribosyl transferase (APRT) deficient cells when labeled adenine is added to the medium (14) and between normal and thymidine kinase (TK) deficient cells when labeled thymidine is added (73).

The incorporation of purine and pyrimidine bases into acid precipitable material can

also be determined by counting the amount of radioactivity in the acid precipitate in a liquid scintillation counter (14,90,23,95). This gives more quantitative results than autoradiography, but information about the difference in labeling of donor and recipient cells is lost, unless these cells can be separated before counting. This is e.g. the case when normal erythrocytes are used as donor cells and which can easily be washed off from the recipient HPRT negative fibroblasts (95). The assays described above do not exclusively measure transfer of either metabolites or of compounds leading to restoration of enzyme activity in the mutant cells and do not discriminate whether metabolic cooperation is caused by transfer of metabolites or of compounds leading to enzyme activity (see also chapter 4).

2.2 *Selective Media*

Corsaro and Migeon (16)used an assay based on the fact that HPRT positive cells can not proliferate in the presence of 6-thioguanine, a substrate for HPRT, while deficient cells cannot incorporate this purine analogue. Due to metabolic cooperation, HPRT deficient cells can also become 6-thioguanine sensitive and the number of surviving HPRT deficient cells depends on the number of HPRT positive donor cells added and on the efficiency of the communication between the two cell types. The number of HPRT positive cells required to prevent the survival of 50% of the HPRT deficient test cells (mean lethal dose) is a measure for the efficiency of the contact. This quantitative assay can be very informative (17), but the choise of cell combinations is limited, because the results are only comparable when the same test cells are used. In this assay too, the phenotypic correction (6-thioguanine sensitivity) can be attributed to both transfer of metabolite (the nucleotide of 6-thioguanine) and restoration of HPRT activity in the test cells.

A selective medium was also used in an assay studying a mutant with elevated PRPP synthetase levels (102). The mutant cells are resistant to 0.2 mM 6-methylmercaptopurine riboside and can transfer this resistance to normal cells by metabolic cooperation. The presence of surviving normal cells after selection can be monitored by measuring the average PRPP synthetase activity in the surviving cells (normal cells lower the average activity). Resistance to the selective medium depends on both inhibitor concentration and PRPP synthetase activity in the cells (102). There is no proof that cells with intermediate enzyme activity are resistant to the inhibitor concentration used. Therefore, this technique is not apt to study transfer of compounds leading to higher

PRPP synthetase activities in the recipient cells. This assay differs from the assay of Corsaro and Migeon, since in the case of the mutated PRPP synthetase the mutant cells act as donors and it is resistance to the selective medium that is passed to the recipient cells.

2.3 *Enzyme Measurements in Individual Cells*

A method that allows quantitative enzyme determinations in individual, isolated cells (44,27), was used to investigate eventual transfer of HPRT or compounds leading to HPRT activity by measuring HPRT activity in individual cells, isolated from a 1:1 mixture of HPRT positive and negative fibroblasts (94). By comparing the distribution of the enzyme activity over the individual cells before and after intimate cell contact conclusions could be drawn about transfer of material leading to HPRT activity in the recipient cell (94). This technique has also been applied by investigators studying contact independent transfer of lysosomal enzymes (78), but the results were less clear because of overlap between activities of normal and mutant cells and because the two cell strains used were labeled by latex particles and indian ink in the lysosomes, which is a dangerous procedure when studying lysosomal enzymes.

2.4 *Preloaded Donor Cells*

When the cells to be used as donors are labeled with a purine or pyrimidine label and washed to remove label, not taken up by the cells, and then brought in contact with the recipient cells, the transfer of metabolites can be monitored. This technique has been applied in a system of normal erythrocytes, preincubated with labeled hypoxanthine, as donor and HPRT deficient fibroblasts as recipient cells (67,95). Appearance of labeled metabolites in the recipient cells can be detected by autoradiography (67) or, because the donor cells can be removed from the recipient cells by washing, by counting in a liquid scintillation counter (95). This technique has also been used with normal cells, preincubated with ^{3}H uridine, as donor and non-preincubated cells as recipient (75). Exchange of labeled compounds was detected autoradiographically. This last assay is not , in contrast to the other assays mentioned here, based upon the phenotypic correction of a mutant cell strain and consequently does not require a cell mutated in an enzyme, of which the activity or the product is demonstrable at the cellular level.

Since metabolic cooperation requires intercellular contact, the phenomenon does not occur in diluted cell cultures. Cessation of metabolic cooperation immediately after dilution of a mixed culture of $HPRT^+$ and $HPRT^-$ fibroblasts, that had been communicating, has been reported (18,73). However, after a similar experiment other investigators found that the phenotypic correction of the mutant cells remained for more than 24 hours (90,35). A likely explanation for this discrepancy is, that the former groups used an incubation time with hypoxanthine of 3 hours before autoradiography (18,73) and the latter incubated longer (35). Subak-Sharpe also found a long half life of metabolic cooperation, although identification of the labeled cells as originally HPRT deficient appeared to be difficult (90 p.289). It is not quite clear what incubation time was used by Subak-Sharpe, but about at the time that these findings were reported in a symposium discussion (90), Subak-Sharpe and coworkers changed from long (93) to short (14) incubation times.

Long incubation times can also lead to metabolic cooperation between cells that are reported to be non-communicators. Migeon, Norum and Corsaro reported that human leukocytes could not correct the mutation in fibroblasts from a Lesch-Nyhan patient after a short incubation time (62) and Cox and coworkers could not detect any metabolic cooperation between either unstimulated or phytohemagglutinin stimulated human lymphocytes (22). Oei and de Bruyn, however, found that unstimulated lymphocytes were taking part in metabolic cooperation with erythrocytes and lymphocytes and these authors used an incubation time of 16 hours (67,28). Certain cancer cells that do not show metabolic cooperation in a normal assay with incubation times of about 3 hours, can communicate after longer incubation times (8). This form of phenotypic correction does not require intercellular contact (8). Also mouse L cells, an often used example of "non-communicating" cells (73,41,19,20), do communicate when the incubation time is longer than 15 hours (20). For this, however, cellular contact appears to be necessary (Cox, personal communication). The so-called selective communicator cells (par.3.3) are incapable of metabolic cooperation with certain cells when short incubation times are used (32,74). These selective communicators lose part of this selectivity when long incubation times are used i.e. they can also communicate with cells that would normally not permit this (74). The classification of communicating, non-communicating and also selectively communicating cells, that is used in the literature, is based upon the results of assays with short incubation times.

Since seemingly contradictory results are obtained when different incubation times are used, the conclusion is justified that experiments using different incubation times are in fact studying different forms of metabolic cooperation.

It is not known, why in the examples given above long incubation times are required. These forms of metabolic cooperation might be less efficient than the others (and need long incubation times to get a detectable incorporation) or the process itself might take a long time, e.g. because it requires the donor or recipient cell to be in a certain phase of the cell cycle.

3 *INFLUENCE OF THE CELL SYSTEM*

3.1 *Cells That Do Exhibit Metabolic Cooperation*

Metabolic cooperation was first reported between polyoma virus transformed hamster fibroblasts (91,14). Until now it is shown to exist between human fibroblasts, both adult (33,20,94,102) and, though less efficient, fetal (16), human fibroblasts with mutations involving membrane functions (16,17), and untransformed hamster fibroblasts (19). In addition, many intra- and interspecies combinations of different cell types have been found to be capable of metabolic cooperation as is illustrated in table I. All these cells were found to be capable of metabolic cooperation after experiments with short incubation times.

Some of the cell combinations mentiones in table I require some special attention. Both enucleated human fibroblasts (23) and human erythrocytes (67,95) can participate as donor in metabolic cooperation with human fibroblasts. Supported by the fact that the protein synthesis inhibitor puromycin has no significant effect on metabolic cooperation (39) and by studies with hybrid cells (15; see par.3.4), this suggests that metabolic cooperation does not require nuclear control or protein synthesis. Apparently the mere presence of a factor or a structure in a cell enables that cell to communicate. The previous suggestion of involvement of the cell nucleus in metabolic cooperation, based upon the finding that efficiency of metabolic cooperation was related to the intracellular location of the transferred compound as detected by autoradiography (20) was later proven to be wrong by more direct evidence (23).

It was shown that in a mixture of cells, mutated in different enzymes, the two subpopulations corrected each other's phenotype (14,73). After prolonged cultivation such a mixed culture regulated its own composition (73).

TABLE I

Survey of Cell Combinations that Show Metabolic Cooperation

Donor	Recipient	References
Polyoma transformed hamster fibroblasts	polyoma transformed hamster fibroblasts	91,14
Hamster fibroblasts	Hamster fibroblasts	19
Human fibroblasts: adult	human fibroblasts[a]	33,20,94,102
fetal	human fibroblasts[a]	16
Human lymphocytes	human lymphocytes[a]	67,28
Human skin biopsy	human skin biopsy[b]	34
Chinese hamster ovary cells	human fibroblasts	16
Hamster fibroblasts	human fibroblasts	19
Mouse fibroblasts	human fibroblasts	19
Polyoma transformed mouse fibroblasts	human fibroblasts	19
Certain HeLa sublines	human fibroblasts	19
Enucleated human fibroblasts	human fibroblasts[a]	23
Human erythrocytes	human fibroblasts[a]	67,95
Human fibroblasts	hamster fibroblasts	19
Mouse fibroblasts	hamster fibroblasts	19
Rat fibroblasts	polyoma transformed hamster fibroblasts	8
Rat liver cells	polyoma transformed hamster fibroblasts	8
Mouse embryo fibroblasts	polyoma transformed hamster fibroblasts	87
Human erythrocytes	human lymphocytes[a]	67,28

[a] See also paragraph 3.1
[b] Tested in vivo.

Until now, no exception has been reported to the general rule that the same celltype, that can act as a donor in metabolic cooperation, can also be a recipient.

3.2 *Cells Incapable of Metabolic Cooperation*

The mouse fibroblast line L929 and its derivatives (shortly "L cells") are not able to participate in metabolic cooperation (73,19), when tested after a short incubation time with labeled substrate before autoradiography. Also some (but not all) HeLa sublines (1) and some cancer cells (8,31) are incapable to act as donor or recipient cells. After short labeling times also leukocytes are incapable of metabolic cooperation (62, 22). Although all these cells can act as donor or recipient cell (20,8,67,28) after prolonged labeling times (see par.2.5), they will on historical grounds be referred to as "non-communicating" cells.

It has been suggested that metabolic cooperation and contact inhibition might be related forms of cell communication (87,6,7). However, since some HeLa sublines (19) and some mammary cancer cells (31) are metabolic cooperators, a normal 3T3 line and a polyoma transformed subline with reduced contact inhibition are equally efficient donor cells (19), and untransformed L cells do not communicate, this hypothesis does not hold.

Absence of metabolic cooperation is also suggested to be related to cell morphology (99,100). However, changing the cell shape of L cells from rounded ("non-cooperating") to more flattened (putative communicating) by treatment with hydrocortisone, cyclic AMP or X-irradiation could not restore metabolic cooperation between cells that were at first inable to communicate (20). Colcemid ($2x10^{-7}$M), which disaggregates microtubuli, did not affect the communication between cells participating in metabolic cooperation (20). However, cytochalastin B (10^{-5}g/l), which breaks down microfilaments, had some disturbing effect on metabolic cooperation (20,16). Omission of serum from the medium has , according to Cox and associates (21), some effect on cell shape, but not on metabolic cooperation. In contrast to that, Corsaro and Migeon (16) found with a quantitative assay an adverse effect of serum omission on metabolic cooperation. This could be due to the bad condition of the cells as a consequence of the serum deprivation (16). Alterations in the medium, such as lowering the Na^+ or Ca^{2+} concentration or raising the osmolarity by adding sucrose, did not change the efficiency of metabolic cooperation (20). Reversion of MEC^- (metabolic cooperation deficient) cells, obtained by selection from a MEC^+ culture, by cyclic AMP (100) can be explained by the hypothesis that the MEC^- cells were selected for a cell shape that hindered intimate cell contact:

altering that cell shape will make intercellular contact and thus metabolic cooperation possible. There are no indications that cell morphology is a more than incidental cause for absence of metabolic cooperation.

3.3 *Selective Metabolic Cooperation*

In 1976 several reports were published about a third class of cells, the "selective communicators" (32,74). Human mammary fibroblasts and human mammary epytheloid (benign tumor derived) cells could communicate with cells from the same cell type, but not with each other (32). Other cell types like rat liver cells and hamster fibroblasts (32,74) also belong to the class of "selective communicators", i.e. they communicate with themselves, with some (but not all) other selective communicators and with all cells that show no selectivity in metabolic cooperation. "Non-selective communicators", like calf lens cells, communicate with themselves and with all other selective and non-selective communicators (see par.3.1). Non-communicators are cells like mouse L fibroblasts that are incapable of metabolic cooperation, even with cells of the same cell type (32; see par.3.2). It should be kept in mind that this classification was made on the basis of experiments with short ($\pm$ 3 hours) incubation times (32,74) and that "selective communicators" show no selectivity when tested with longer incubation times (74).

It is to be expected that the group of selective communicators consists of two (or more) subgroups of cell types, that communicate with all other cell types of the same subgroup and do not do so with cells from another subgroup. From the results thus far published (32,74,31) it can be concluded that selective communicators exhibit metabolic cooperation with identical cell types, with cells from the same subgroup and with non-selective communicators with diminishing efficiency.

Interesting in this connection is the finding that human mammary epitheloid cells that are transformed (i.e. from a cancerous tumor) lose their specificity in metabolic cooperation and become either non-communicating or non-selectively communicating (31). It has been suggested that transformation is associated with loss of either the ability to communicate or of selectivity (31). It fits well in the previous findings that sometimes transformed cells do and sometimes do not show metabolic cooperation (see par.3.2).

It should be kept in mind that cells previously described as communicating, might in fact be selective communicators, that have not been tested yet for metabolic cooperation with a selective communicator of a different subgroup.

3.4 *Hybrid Cells*

To investigate the mechanisms underlying metabolic cooperation, communicating and non-communicating cells have been fused with the aid of inactivated Sendai virus. The resulting heterokaryons are able to take part in metabolic cooperation, so the ability to communicate is genetically transferred as a dominant trait (62). It has also been shown that hybrid cells are able to act as donor soon after fusion, before the cell nuclei have been reorganized (15). Apparently metabolic cooperation does not require the control of a nucleus (see also par.3.1).

Cellular hybridisation techniques can be useful tools in elucidating the mechanisms of metabolic cooperation, e.g. by fusion experiments with selective communicators.

4 *TRANSFERRED MATERIAL*

4.1 *Enzymes Involved*

The phenotype of many mutants can be corrected by transfer of material from cell to cell. The phenotypic correction of cells with a deviant mucopolysacharide metabolism (66) or with a mutated hexosaminidase (28) does not fit the stringent definition of metabolic cooperation formulated by Subak-Sharpe et al. (92), because physical contact between mutant and normal cell is not required (see also par.1.1). It is interesting, that this form of phenotypic correction could not be demonstrated with cells with a mutant α-glucosidase or β-galactosidase (28), two other lysosomal acid hydrolases.

True metabolic cooperation has been reported for the enzymes HPRT (91,87,33,18,76, 94), APRT (14,19), PRPP synthetase (102), TK (73) and deoxycytidine kinase (99). Glucose-6-phosphate has been shown to migrate from cell to cell (49). This suggests that 6-phosphoglucono-δ-lactone or 6-phosphogluconate, reaction products of the glucose-6-phosphate dehydrogenase (G-6-PD) reaction and compounds related to glucose-6-phosphate, are also exchangeable, resulting in metabolic cooperation for G-6-PD. It has been shown that the enzyme G-6-PD itself is not transferred from normal to mutant cell (19), but a mutant phenotype can also be corrected by transfer of enzyme products (see also par.4.2).

4.2 *The Nature of the Transferred Material*

Phenotypic correction of a mutant cell during physical contact with a normal cell can theoretically be the result of transfer of the following substances(92):

1. Reaction product of the enzyme under study or a related compound (e.g. for metabolic cooperation between HPRT positive and HPRT negative cells IMP or a related compound).
2. Macromolecules with the reaction product incorporated (in the example with HPRT, polynucleotides).
3. Informational polynucleotides (mRNA or episomal DNA) coding for the enzyme that is missing in the recipient cell.
4. Intact enzyme or subunits.
5. A regulatory molecule for the missing enzyme.

These possibilities are grouped into two classes, that will be discussed in detail: a) Transfer of low molecular weight metabolites. b) Transfer of macromolecules leading to enzyme activity in the mutant cell.

It will be clear that, when e.g. mRNA coding for HPRT is transferrable, this implies that polynucleotides with the reaction product IMP incorporated can also be transferred. For other enzymes of nucleic acid and protein metabolism analogous remarks can be made.

4.3 *Low Molecular Weight Metabolites*

Much of the evidence presented for the exchange of metabolites is indirect. Mixtures of normal and mutant cells, that show metabolic cooperation when grown in dense cultures, cease doing so when they are diluted. This reversion of the phenotypic correction is reported to happen almost instantaneously after dilution (18,73,102) in studies that use a 3 hour incubation time. The correcting influence has a very short half life, shorter than the enzyme under study (18). If the mutant cells receive a compound leading to reversion to normal enzyme activities, these cells would behave as normal cells, even after cell contact is interrupted. Therefore, the short half life of the correction is an argument supporting the hypothesis of metabolite exchange.

Direct evidence for this has also been given. When donor cells are labeled before contact, the recipient cells appear to take up label during contact with these labeled donor cells. In this way transfer of IMP (20) and of uridine nucleotide (75) or a related compound has been shown. Besides these studies with short labeling times, the parallel experiment with preloaded erythrocytes, that needs a 16 hour incubation time, also

presents evidence for the transfer of metabolites (68,95). Erythrocytes, incubated with ^{3}H labeled hypoxanthine, have the label almost exclusively in the form of IMP, so it is very likely that, at least in these expeiments, the transferred compound is IMP itself and not a related compound (68,95).

4.4 *Molecules Leading to Enzyme Activity*

The same turnover determination of the corrective effect, that is indicative for transfer of metabolites (see par.4.3), has also given results that suggest transfer of a compound leading to enzyme activity (90,35). In the latter studies long incubation times were used (see also par.2.5). It was shown with autoradiographic studies that HPRT deficient fibroblasts in a mixture of HPRT positive and HPRT negative fibroblasts still behave phenotypically normal more than 24 hours after intercellular contact had been interrupted by dilution (90,35). The mutant cells apparently retained the ability to incorporate radioactive hypoxanthine, i.e. they could perform the HPRT reaction.

Other indirect evidence for the transfer of substances, that enhance enzyme activity in the recipient cells, is derived from (semi)quantitative, autoradiographic studies. A mixed culture of HPRT positive and HPRT negative fibroblasts incorporate together, under conditions allowing metabolic cooperation, about as much hypoxanthine as the HPRT positive cells, present in the mixture, would do alone (90,14,23). Apparently, the HPRT positive cells take up the hypoxanthine to convert it into IMP, that is subsequently distributed over the cell population; uptake of hypoxanthine or conversion into IMP in the HPRT positive cells is apparently the rate limiting step (14,23).

In a 1:1 mixed culture of APRT positive and APRT negative cells, however, all cells together incorporate more adenine than the APRT positive cells alone can account for (90,14). In addition, not only that half of the cell population with the lowest number of grains per cell (assumed to be APRT negative cells) show more grains per cell after autoradiography than they would have when cultured apart, but also the APRT positive cells have more grains per cell than they have when cultured apart (90). Apparently, incorporation is not only stimulated in the enzyme deficient cells by contact with the APRT positive cells, but also in APRT positive cells by APRT negative cells (90). This cannot be explained by transfer of metabolites, because the APRT negative cells cannot form anything that the APRT positive cells are unable to make. Besides adenine nucleotides, also other compounds are exchanged.

Transfer of informational molecules as an explanation for metabolic cooperation was

studied directly by Uitendaal et al. (94) using quantitative enzyme measurements in single, isolated fibroblasts (44,27). HPRT positive and HPRT negative fibroblasts, when grown in different dishes, form two different populations concerning HPRT activity per cell. A 1:1 mixture of those cells, however, grown in close contact for 72 hours and then separated, could not be divided into two populations on the basis of their HPRT activities. The cells from the mixture showed a broad spectrum of HPRT activities with the mode on intermediate activity (94). This is illustrated by the following figures. From the separately grown $HPRT^+$ and $HPRT^-$ strains in total only 18% had intermediate activities (between 0.3 and 0.9 x 10^{-13}moles/cell.hr). From the mixed culture 51% was in this range. The mean activities were 0.07 x 10^{-13} and 1.22 x 10^{-13} moles/cell.hr for the $HPRT^-$ and the $HPRT^+$ strains, respectively (unpublished results). The enzyme activity in the cells was determined after intercellular contact had been broken by dilution and after the cells had been further cultured for eight hours (94). These results can only be explained by assuming that the mutant cells had acquired HPRT activity during contact with the HPRT positive cells, i.e. that a compound leading to HPRT activity had been transferred.

Evidence for exchange of RNA between 3T3 mouse cells has been presented by Kolodny (50,51). Donor cells were prelabeled with ^3H uridine, that was incorporated into RNA, before contact with unlabeled recipient cells. Separation of donor and recipient cells was posible with gravitation force, because the donor cells had ingested tantalum particles (50). After contact and separation, the recipient cells also appeared to have radioactive RNA. Transfer of low molecular weight degradation products and incorporation during RNA synthesis in the recipient cells was excluded by the use of inhibitors of DNA dependent RNA synthesis, analysis of pyrimidine bases in RNA and the use of labeled methionine (50). In analogous experiments, transfer of protein could be shown to occur (51), while DNA appeared to be not transferrable from cell to cell (50). The protein transfer itself was also selective; of the nuclear protein the histones were transferred preferentially (51). These results have been contradicted by a study by Pitts and Simms (75). A valid explanation for the difference in results could not be given; failure of Kolodny's separation of donor and recipient cells, as suggested by Pitts and Simms (75), is not the reason for conflicting results. In that case, Kolodny would also have found labeled DNA in his recipient cell fraction, which he did not (50).

In the squid, the length of giant cells (neuron cells with axons of 15 to 25 cm) poses problems concerning transport of protein from the cell body, where they are synthesized, to the end of the axons. This is overcome by transfer of protein from sur-

rounding Schwann cells to the axon (55,36). It has been estimated that the amount of protein in the axon that comes from the Schwann cells is about five times the amount of protein that is synthesized in the giant cell body and transported to the axon (55). Transfer of radioactive precursors from Schwann cells to axon and incorporation of these during protein synthesis in the axon as an explanation of the radioactive protein in the axon, is excluded by several arguments: a) the isolated axon contains hardly any ribosomal RNA; b) axoplasma has no capacity to incorporate radioactive leucine; c) injection or perfusion of the axon with RNase does not inhibit the phenomenon (55,36). Since the SDS polyacrylamide profiles of labeled proteins in donor and recipient cells differ, it was concluded that the transfer is a selective process (55).

Of course it can be objected that the protein transfer reported here is measured in a very specialized cell system that cannot depend on its own protein synthesis, but, as mentioned before, transfer of RNA and protein also occurs in 3T3 mouse fibroblasts (50, 51,52). This suggests that protein transfer might be a more general phenomenon. Anyhow, passage of proteins across membranes has been reported before (13). The studies about transfer of macromolecules strongly support the reports mentioned in the beginning of this paragraph (90 p.289,35,94), that favour exchange of molecules leading to enzyme activities as a basis for metabolic cooperation.

5 *MECHANISMS FOR TRANSFER*

5.1 *Gap Junctions*

When searching for structures that permit passage of substances causing phenotypic correction, one will find gap junctions a very likely possibility.

Gap junctions are hexagonal structures on membranes of a great variety of cell types that connect adjacent cells with channels, while leaving between the cells a gap of 20-30 Å, that is accesible to extracellular molecules such as colloidal lanthanum hydrochloride, as can be seen under an electron microscope (for reviews, see 12,60,86,97). They are believed to allow passage of ions and fluorogenic dyes (37,47,77,86,97). Gap junctions can be isolated and purified (11,41,42,40). Purified preparations contain approximately 50% protein, 7% cholesterol and 40% phospholipid. The protein can be resolved by SDS polyacrylamide gel electrophoresis into only two components (37,40).

5.2 *Relation between Gap Junctions and Metabolic Cooperation*

In 1972 a positive correlation has been reported between metabolic cooperation and gap junctions (38). During the same year, such a correlation has also been found between on the one hand, metabolic cooperation and, on the other, ionic coupling and dye exchange (8), believed to go through gap junctions (37,47,77,86,98,97).

Gap junctions and metabolic cooperation (tested with a short incubation time; see also par.2.5) have many properties in common. Mouse L cells cannot form gap junctions (8,5,37); they also cannot participate in metabolic cooperation when assayed the normal way (short incubation time)(73,19). Hybrids between cells capable and incapable of making gap junctions can form gap junctions (6,5); participation in metabolic cooperation is also inherited as a dominant trait in fusion experiments (see par.3.4). Formation of gap junctions does not require de novo protein synthesis (81,30), neither does metabolic cooperation (67,23,95; see par.3.1). Neither gap junctions (76),nor metabolic cooperation (17) are coupled with ouabain sensitive ATPase, another membrane funtion. Just as is known for metabolic cooperation (19), gap junction formation has been reported to be insensitive to energy metabolism inhibitors (30), although an opposite result regarding this point also has been published (76). Neither gap junctions (41), nor metabolic cooperation (20) are affected by a treatment of the cell under study with proteolytic enzymes. The effect of several other treatments of the cell membrane upon metabolic cooperation has also been investigated. Enzymatic treatment of the communicating cells with neuraminidase did not influence metabolic cooperation (20). Sulphhydryl blocking agents (0.1 M p-chloromercuribenzoate or 1.0 mM N-ethylmaleimide)(19) and urea (20) also had no effect upon metabolic cooperation. The only treatment known thus far to influence both cell membrane and metabolic cooperation is oxidation by peroxidase (20). The effect of these treatments upon gap junctions is not yet known.

An important reason why gap junctions are believed to be involved in metabolic cooperation is the fact that they are permeable for compounds with molecular weights up to 1200. This is comparable with the molecular weight of metabolites that are believed to cause the phenotypic correction in metabolic cooperation (see below).

5.3 *Permeability of Gap Junctions*

It is generally accepted that gap junctions are permeable not only to small ions like Na^+, K^+,Cl^- etc. (as measured with microelectrodes placed in neighbouring cells),

but also to a variety of microscopic markers such as fluorescein (molecular weight ± 330) and procion yellow (mol.wt. ± 625) (see e.g.12,97).

Reports about permeability for high molecular weight compounds are somewhat contradictory. In 1971 Reese, Bennett and Feder reported that microperoxidase (mol.wt. 1800), but not horse radish peroxidase (HRP; mol.wt. 44,000) could pass through gap junctions, as seen under an electron microscope (77). In 1973, however, Bennett reported that the aldehyde fixation used seems to enhance the permeability of gap junctions for high molecular weight compounds (12). On the other hand, Goodenough and Revel found that HRP could penetrate from the inside of the cell into the 20 Å gap of a gap junction, probably in the intercellular channels, while beef catalase (mol.wt. 240,000) could not (41). In addition, *Clostridium welchii* phospholipase C (mol.wt. 42,000) also had access to the gap, i.e. the enzyme could alter the morphology of the gap, starting from the inner surface of the junction (41). Since enzymatic alteration was performed before fixation, diffusion of phospholipase C into the 20 Å gap cannot be explained as an artefact of fixation, described by Bennett et al. (12). Part of these experiments have been performed with isolated gap junctions, but these gap junctions were morphologically unaltered. Therefore, the possibility that compounds with a molecular weight up to 44,000 can pass through in vivo existing channels in gap junctions is far from unlikely (41). In 1966 Kanno and Loewenstein reported that, when serum albumin coupled to a fluorescent dye (mol.wt. 69,000) was injected into a *Drosophila* salivary gland cell, a fluorescent compound could be seen in the adjacent cells with the antigenic properties of serum albumin and with a diffusion rate corresponding to molecular weights of at least 10^4 (48). However, further experiments convinced Loewenstein's group that the molecular weight of molecules that still could pass through gap junctions was considerably lower (58,82). In 1977, Simpson, Rose and Loewenstein tested the permeability of gap junctions for a broad scala of compounds with different molecular weights and found that the upper limit was between 1200 and 1900 (82). Apparently, in their experiments in 1966 a fluorescent-labeled fragment, still carrying antigenic determinants of the original molecule, passed through the junctions. The information upon junctional transit speeds for large peptide molecules has greatly increased since 1966 and leads to the conclusion that at that time overestimates for the molecular weights were made (Loewenstein, personal communication).

However uncertain the upper limit of the molecular weight for a passable molecule is, there is no doubt that hypoxanthine, adenine, thymidine and uridine nucleotides, the metabolites that are believed to be transferred during metabolic cooperation (see

par.4.3), fall with respect to their molecular weight in the range of molecules that can pass from cell to cell through gap junctions. With respect to informational macromolecules it can only be stated that, if they are transferred from cell to cell during metabolic cooperation, transfer through gap junctions cannot be excluded.

5.4 *Metabolic Cooperation and Gap Junctions Are Not Mutually Interdependend*

5.4.1 Absence of metabolic cooperation in the presence of gap junctions

Unstimulated lymphocytes are not ionically coupled, but lymphocytes stimulated with mitogenic lectins are (45,46,68), i.e. they have gap junctions (3,71). However, neither unstimulated, nor stimulated lymphocytes can participate in metabolic cooperation, when assayed with a short incubation time (22). Hybrids derived from communicating fibroblasts and non-communicating L cells were shown to have gap junctions and to be ionically coupled; also fluorescein could be transferred from cell to cell (5). (Fluorescein transfer is indicative of the possibility to exchange low molecular weight metabolites; see par.5.3). However, after some of the human chromosomes were lost, some of the hybrids retained ionic coupling and gap junction formation, but were unable to exchange fluorescein (5,7). These cells showed, instead of gap junctions, special intramembranous fibrillar structures in their junctions (54).

Ionically coupled cells of teleost embryos and of xenopus blastulae (84) also could not exchange fluorescein. Of course in the last two cases the cells used are very different from those normally used for metabolic cooperation studies, but they support the conclusion that can be drawn from the other examples, i.e. the presence of gap junctions does not necessarily implicate metabolic cooperation.

5.4.2 Intercellular transport in the absence of gap junctions

As already demonstrated in paragraph 3.2 some putative non-communicating cells (L cells, unstimulated lymphocytes) can act as donor or recipient cells in phenotypic correction when long incubation times, longer than 15 hours, are used before autoradiography (8,20,67). It has been demonstrated that these cells, or at least the great majority of the cells, are not ionically coupled and do not form gap junctions (45,38, 8). Since almost all cells in these experiments did participate in phenotypic correction (20,67), at least a considerable part of them must have done so without gap junctions. It should be kept in mind, that in the case of communicating lymphocytes (67) the use of a mixture of lymphocytes might lead to stimulation of the cells by a mixed

lymphocyte reaction and stimulated lymphocytes form gap junctions (45,46,69). However, a long incubation time is required to detect the phenotypic correction (67) and that generally implies that gap junctions are not involved (see par.5.5). Besides, the L cells clearly communicate without the presence of gap junctions. The possibility of this is further supported by the fact that glucose-6-phosphate can be exchanged by the gap junctions-lacking L cells (49). In addition, lowering the Ca^{2+} concentration in the culture medium diminished ionic coupling and the number of gap junctions (3,71), while it had no effect upon metabolic cooperation (20).

So, intercellular transfer of compounds that influence metabolism does not necessarily require gap junctions.

It is very doubtful if erythrocytes can form gap junctions.Therefore, metabolic cooperation between IMP loaded erythrocytes and $HPRT^-$ fibroblasts, that does need long incubation times, is probably another example of metabolic cooperation in the absence of gap junctions (67,28,95).

5.5 *Gap Junctions, Metabolic Cooperation and Incubation Times*

The answer to the question of whether there is a relation between metabolic cooperation and gap junctions depends partly on the way metabolic cooperation is detected. Except for the cases mentioned in paragraph 5.4.1, cells with gap junctions exhibit metabolic cooperation as measured with autoradiography after a short incubation time (e.g.8,38). On the other hand, several examples have been found of cells that do participate in metabolic cooperation without gap junctions (par.5.4.2), when long incubation times are applied.

Studies that found an immediate termination of the correcting effect after dilution of the mixed, metaboliccaly cooperating fibroblast cultures (81,73,75), i.e. studies that dealt with transfer of metabolites, all used short labeling times of 2 to 3 hours. Since in this cell system demonstration through gap junctions is also possible after a short labeling time (8,38), it is generally assumed that in these cases metabolic cooperation is caused by transfer of metabolites through gap junctions. This does no imply that, whenever metabolites are transferred, gap junctions are involved. In metabolic cooperation between erythrocytes and fibroblasts, for example, IMP can be transferred without the presence of gap junctions, but then long incubation times are needed (67,28,95). In this case IMP might be transported in a way similar to the transfer of glucose-6-phosphate between the gap junctions-lacking L cells (50). Nevertheless,the

relationship between gap junctions, metabolic cooperation by transfer of metabolites and short incubation times is very clear. It may even sometimes lead to the erroneous assumption that transfer of metabolites through gap junctions is the only form of metabolic cooperation, because autoradiography after 3 hours labeling is the most often used assay for metabolic cooperation.

5.6 *Mechanisms for Transfer of Macromolecules*

Whether macromolecules can be transported through gap junctions is not known. When gap junctions are permeable for enzymes with a molecular weight even higher than 40,000 (41), then they are certainly permeable for the subunits of several enzymes such as HPRT. The HPRT subunits in their smallest form with a molecular weight of 26,000 (69, 89, 64,70), may themselves have no HPRT activity but can reaggregate to active subunits (89). A non-mutated subunit may correct a whole mutant HPRT molecule in a way similar to that when a normal enzyme can render mutant HPRT active again under special conditions (10). Neither the enzyme G-6-PD,however, nor its subunits are transferred or, if they are, do not lead to G-6-PD activity in the recipient cells (19). Also the transfer of isocitrate dehydrogenase or its subunits was below detection level (63).

Direct intercytoplasmic exchange of macromolecules not through gap junctions, such as is found for the metabolite glucose-6-phosphate (49), is an alternative.

Exchange of protein might also take place between membranes of apposed cells. In this context the demonstration of HPRT in cellular membranes (26) could be very important. Identification of donor and recipient cells in a metabolic cooperation experiment by labeling the membranes with antisera failed, because the cells appeared to exchange the membrane bound antigens (73).

Mere excretion of protein into the medium by donor cells and subsequent uptake by the recipient cells seems to play a minor role, if at all; HPRT deficient fibroblasts are not reverted by addition of HPRT containing celllysate to the medium (95), unless very soon after trypsinisation (4). Apparently, permeability of the cell membrane is affected by the trypsin treatment.

Pinocytosis of parts of the neighbouring cells is also mentioned as a mechanism of macromolecular exchange (43).

6.1 *Different Types of Metabolic Cooperation*

Evidence has been presented that transfer of both metabolites and compounds leading to enzyme activity can be the basis of metabolic cooperation (see par.4.2 and 4.3). In addition, studies showing intercellular transfer of macromolecules not within the context of metabolic cooperation have been published (par.4.3). Therefore, the conclusion can be drawn that there are at least two forms of metabolic cooperation.

Although the relationship between metabolic cooperation and gap junctions has clearly been established (see par.5.2), many cases are known that do not fit into this relationship (see par.5.4). Therefore again the conclusion is justified that there are different forms of metabolic cooperation.

Apparently contradictory results can be explained because different assays for metabolic cooperation have been used (chapter 2). Of major importance appears to be the incubation time used with labeled substrates before autoradiography (par.2.5). The use of different incubation times leads to the investigation of at least two forms of metabolic cooperation.

It is evident that it mainly depends on the cell system under study which type of metabolic cooperation is investigated. This point is discussed in detail in chapter 3.

After this compilation of arguments, the conclusion that there are different types of metabolic cooperation is inevitable (see also 94,28,95).

A hypothetical classification of the different forms, described in the literature can be made. It should be kept in mind, however, that this list may be incomplete and that, on the other hand, since only certain aspects of the reported forms of metabolic cooperation are mentioned in the respective publications, some forms listed may be identical. In addition, some forms of intercellular contact are perhaps not metabolic cooperation in the strict sence, because they might not require cellular contact(par.1.1).

6.2 *Transfer of Metabolites through Gap Junctions*

There is no doubt that metabolites, such as IMP, can be transferred from cell to cell (18,73,67,75,95). As is discussed in paragraph 5.5, the metabolites probably pass through gap junctions and this type of metabolic cooperation can be detected autoradiographically after a short labeling time of about 3 hours.

6.3 *Transfer of Metabolites from Erythrocytes and Unstimulated Lymphocytes*

Transfer of IMP has been shown to occur from erythrocytes to fibroblasts (67,95) and unstimulated lymphocytes (67) and from unstimulated lymphocytes to unstimulated lymphocytes(67). Unstimulated lymphocytes are not ionically coupled (45,46), so they have no gap junctions (3,71). Gap junction formation by erythrocytes has also never been found. Therefore, it is concluded that this type of metabolic cooperation differs from the one mentioned above. This type requires long incubation times before autoradiography (67,95).

6.4 *Transfer of Metabolites between "Non-Communicating" Cells*

Mouse L fibroblasts, that lack gap junctions and are incapable of ionic coupling and fluorescein transfer (8,38,49), exchange glucose-6-phosphate (49). Simple leakage through damaged membranes is excluded, because glucose-6-phosphate transfer happens much more efficient than fluorescein transfer (49). This also indicates that the transport, whatever its mechanism is, is selective. Since the process requires cellular contact (49) and glucose-6-phosphate is a very important metabolite, it is an example of true metabolic cooperation (see par.1.1).

Since the glucose-6-phosphate transfer seems to go very rapidly (49) and, on the other hand, IMP transfer from erythrocytes and lymphocytes requires long incubation times (67,95), these two forms of metabolite transfer in the absence of gap junctions might be based on different mechanisms. However, the detection methods are so very different that this kind of comparison might be impossible.

Interestingly, transfer of glucose-6-phosphate between L cells is inhibited by the energy metabolism inhibitor oligomycin (49).

6.5 *Transfer of Compounds Leading to Enzyme Activity*

After contact with normal fibroblasts, HPRT deficient fibroblasts can acquire HPRT activity (94). Reversion of HPRT deficiency in the recipient cell has also been suggested on the basis of more conventional studies regarding the half life of the phenotypic correction as a result of metabolic cooperation (90,35). This seems, however, not to happen when the donor cells are erythrocytes (95). The nature of the transferred compound has not yet been established. According to Kolodny, both high molecular weight RNA (50) and protein (51), but not DNA (50) can be exchanged between mammalian fibro-

blasts in culture. Transfer of protein has also been proven to occur from Schwann cells to the giant cell axon in the squid (55,36). Therefore, enzymes, subunits or mRNA coding for enzymes can be the compounds that are transferred during intercellular contact. The mechanisms by which these informational molecules are transferred are not known. In paragraph 5.6 several possibilities are discussed. Detection of this type of metabolic cooperation requires long labeling times (90,35).

6.6 *Transfer of Unidentified Compounds between "Non-Communicating" Cells*

Mouse L cells and "non-communicating" cancer cells can act as donor or recipient cell in metabolic cooperation (8,20; see also par.2.5). This is only detectable after long incubation times. The nature of the transferred material is not known; there is even no indication whether it has a high or a low molecular weight.

One report specifically mentions that this form of phenotypic correction does not require intercellular contact (8); it is therefore not metabolic cooperation in the strict sence. However, these results can easily be confused with the other forms of metabolic cooperation listed elsewhere in this chapter. The phenotypic correction of L cells (20) does require intercellular contact (Cox, personal communication) and therefore fits into the definition of metabolic cooperation given in paragraph 1.1.

If the transferred compound will later appear to be a metabolite, then this form of metabolic cooperation may be identical to the ones mentioned in paragraph 6.3 and 6.4. On the other hand, it may also later appear that in these "non-communicating" cells informational macromolecules are transferred.

6.7 *Uptake of Enzymes from the Medium*

This form of phenotypic correction is not metabolic cooperation, since it does not require physical contact between donor and recipient cell. It is mentioned here, because it can easily interfere in studies about metabolic cooperation: it can occur in mixed cultures of normal and mutated cells and phenotypic correction is effectuated by transfer of compounds leading to enzyme activity (i.e. the enzyme itself) from donor to recipient cell.

Excretion of enzymes by cultured fibroblasts and subsequent uptake of these enzymes into lysosomes of other fibroblasts in the same culture is quite a normal procedure for certain lysosomal enzymes (66,78) and might even be the normal way for fibroblasts to

transport these enzymes after synthesis into the lysosomes.

Uptake of enzymes from the medium into fibroblasts has also been reported for HPRT added to the medium as lysate of normal cells (4). However, this appeared to be an artefact, since the efficiency of HPRT uptake was inversely proportional to the time passed between the last trypsinisation and the addition of cell lysate (4).

7 *SUMMARY*

In this review a special form of phenotypic interaction, namely metabolic cooperation, is discussed. The normal definition of metabolic cooperation is discussed. This definition is used, i.e. the process whereby the metabolism of cells in contact is modified by exchange of material, although several interesting related phenomena are thus arbitrarily excluded, because they do not require intercellular contact.

From the data in the literature it can nevertheless be concluded that several different phenomena fit into the definition of metabolic cooperation. Which type of metabolic cooperation is studied, strongly depends on the method used, especially on the incubation time with labeled substrates before autoradiography. It also depends on the cell system used.

Both metabolites and compounds leading to enzyme activity can be transported from cell to cell. Metabolites can be transferred through gap junctions, but also other transport mechanisms exist. It is not yet clear in what way the informational molecules can be transferred, but their passage through gap junctions cannot be excluded.

A putative classification of the types of metabolic cooperation investigated is given.

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SUMMARY

This thesis deals with studies on several enzymes of the purine salvage pathway in cultured human fibroblasts. Many of these studies have been performed with ultramicrochemical methods enabling enzyme activity measurements in a small, counted number of cells. These techniques offer many advantages, of which the following were important to the studies compiled in this thesis: Favourable signal:noise ratios can be obtained, enabling enzyme measurements in the 10^{-16}moles/hr range (see e.g. paper 6). Because the enzyme activities are related to the number of cells and not to the amount of protein, the method is very suitable for studies on gene expression (paper 5). The use of lyophilisation to prepare cell lysates has a less damaging effect on enzymes than other techniques and therefore, the method is very apt to do characterisation of especially mutant enzymes with low stabilities (paper 1,4). The fact that enzyme activities in individual cells from a heterogeneous population can be determined allows certain experiments that would otherwise be impossible (paper 7).

The wide applicability of the ultramicrochemical enzyme measurements is illustrated by the fact, that the results obtained with ultramicrochemical characterisation of adenosine deaminase, hypoxanthine phosphoribosyl transferase (HPRT) and purine nucleoside phosphorylase were comparable with data from the literature (paper 2,3,4). In addition, some yet unknown data were found with these methods, such as the substrate inhibition of purine nucleoside phosphorylase by hypoxanthine (paper 3) and the inhibition of 5'-nucleotidase, a very disturbing enzyme in assays for HPRT, by PRPP. Since purine nucleoside phosphorylase is stimulated by its substrate inosine and inhibited by the substrate for its reverse reaction, hypoxanthine, a regulatory role for this enzyme in the purine metabolism in fibroblasts is suggested (paper 3).

Differences of HPRT expression in different HPRT deficient gout- and Lesch-Nyhan-patients are reported. It appears, that there is no clear correlation between the severity of the enzyme deficiency and the clinical symptoms. In addition, there were marked differences in the residual activities of different tissues from one gouty patient. The HPRT of that patient has been characterized in fibroblasts (that have normal activity) and in erythrocytes (that were severely deficient). The only difference with normal HPRT found was a decreased heat stability in both erythrocytes and fibroblasts from the patient (paper 4). Fluctuations in adenosine deaminase expression in

cultured fibroblasts, as clearly registrated ultramicrochemically, can probably be contributed to yet uncontrolable shifts in isozyme patterns (paper 5). Another, though artificial, change in expression of purine salvage enzymes is caused by mycoplasma contamination. A simple, biochemical test for the presence of mycoplasma contamination, based upon the high activity in prokaryotes and the extremely low activity in eukaryotes of the enzyme adenosine phosphorylase, has been improved with respect to sensitivity and adapted to enable parallel tests in the same cell culture dish, that is used for ultramicrochemical experimental or diagnostical purposes (paper 6).

A special form of seeming reexpression of HPRT in $HPRT^-$ fibroblasts is phenotypic correction by metabolic cooperation. It is concluded from shifts in the HPRT activity distribution in individual $HPRT^+$ and $HPRT^-$ fibroblasts, caused by physical contact with eachother, that metabolic cooperation between cultured fibroblasts can be effectuated by transfer of compounds that can lead to enzyme activity in the recipient cell (paper 7). The hypothesis that there are at least two forms of metabolic cooperation, transfer of metabolites and transfer of compounds leading to enzyme activity, is illustrated by results obtained with the erythrocyte-fibroblast and lymphocyte-lymphocyte and with the fibroblast-fibroblast cell system (paper 8). Only one form, transfer of metabolites, occurs in the IMP loaded erythrocyte-fibroblast system. Intact IMP loaded erythrocytes are equally efficient as IMP donors as lysed IMP loaded erythrocytes. The amount of IMP incorporated into the fibroblasts is dependent on the amount of preloaded erythrocytes added (paper 9). Finally, these findings are placed against the background of the reports in the literature about metabolic cooperation. These reports are often dealing with different types of metabolic cooperation, mainly because different detection methods have been used. A tentative classification of the different forms of metabolic cooperation, that have been studied thus far, is given (paper 10).

SAMENVATTING

In dit proefschrift worden onderzoekingen besproken naar enzymen van de purine salvage stofwisseling in gekweekte menselijke fibroblasten. Verscheidene van deze onderzoekingen zijn gedaan met behulp van ultramicrochemische methoden. Deze methoden maken het mogelijk enzymactiviteiten in een klein, geteld aantal cellen te bepalen. Dit biedt vele voordelen, waarvan de volgende van belang waren voor de in dit proefschrift verzamelde onderzoekingen: Een hoge signaal:ruis verhouding kan worden verkregen, zodat enzymactiviteiten in de orde van 10^{-16}mol/uur kunnen worden gemeten (zie b.v. artikel 6). Aangezien de enzymactiviteiten worden uitgedrukt per cel en niet per hoeveelheid eiwit, is de methode zeer geschikt voor onderzoekingen op het gebied van genexpressie (artikel 5). Cellysaten worden gemaakt door middel van lyophiliseren en dat is minder nadelig voor enzymen dan andere technieken, zodat vooral gemuteerde enzymen die minder stabiel zijn goed met de ultramicro-methode kunnen worden gekarakteriseerd (artikels 1 en 4). Doordat enzymactiviteiten in individuele cellen uit een heterogene celpopulatie gemeten kunnen worden, worden bepaalde zeer specifieke experimenten mogelijk (artikel 7).

De uitgebreide toepassingsmogelijkheden van de ultramicrochemische enzymbepalingen wordt geillustreerd door het feit dat de ultramicrochemische karakterisering van adenosine deaminase, hypoxanthine phosphoribosyl transferase (HPRT) en purine nucleoside phosphorylase resultaten gaf, die vergelijkbaar waren met literatuurgegevens hierover (artikels 2, 3 en 4). Bovendien werden enige nog niet bekende bevindingen gedaan met deze techniek, zoals substraatremming van purine nucleoside phosphorylase door hypoxanthine en de remming door PRPP van 5'-nucleotidase, een enzym, dat storend werkt bij HPRT bepalingen. Purine nucleoside phosphorylase wordt door het substraat inosine gestimuleerd en door het substraat voor de omgekeerde reactie, hypoxanthine, geremd. Dit suggereert, dat dit enzym een regulatoire rol speelt in de purine stofwisseling (artikel 3).

Er zijn verschillen in HPRT expressie gevonden tussen verschillende HPRT-deficiente Lesch-Nyhan- en jicht-patienten. Er blijkt geen duidelijk verband te bestaan tussen de mate van HPRT deficientie en het klinische beeld. Ook blijken er verschillen te bestaan tussen de residuele HPRT activiteiten in verschillende weefsels van één jichtpatient. Het HPRT van deze patient is gekarakteriseerd in fibroblasten, die normale activiteit

vertonen, en in erythrocyten, die zwaar deficient zijn. Het enige gevonden verschil met normaal HPRT is een verlaagde hittestabiliteit in zowel erythrocyten als fibroblasten van de patient (artikel 4). Fluctuaties in expressie van adenosine deaminase in gekweekte fibroblasten konden ultramicrochemisch duidelijk worden aangetoond. Dit verschijnsel kan waarschijnlijk worden verklaard door het optreden van tot nu toe oncontroleerbare verschuivingen in het isozym patroon (artikel 5). Nog een voorbeeld van, weliswaar artificiele, verandering van expressie van purine salvage enzymen is die, welke wordt veroorzaakt door contaminatie van de celkweek door mycoplasma. Een eenvoudige biochemische test voor aanwezigheid van mycoplasma contaminatie, gebaseerd op hoge en uiterst lage adenosine phosphorylase activiteit in respectievelijk prokaryoten en eukaryoten, is verbeterd wat betreft gevoeligheid. Bovendien is de test zodanig aangepast, dat dezelfde kweekdish, die voor ultramicrochemische experimenten of diagnoses wordt gebruikt, op de aanwezigheid van mycoplasma kan worden gecontroleerd (artikel 6).

Een speciale vorm van schijnbare herexpressie van HPRT in $HPRT^-$ fibroblasten is fenotypische correctie door metabole cooperatie. Uit verschuivingen in de HPRT verdeling over individuele $HPRT^+$ en $HPRT^-$ fibroblasten na contact met elkaar kan worden geconcludeerd, dat metabole cooperatie het gevolg kan zijn van overdracht van stoffen die kunnen leiden tot enzymactiviteit in de "recipient" cel (artikel 7). De hypothese dat er minstens twee vormen van metabole cooperatie zijn, namelijk overdracht van metabolieten en van stoffen die kunnen leiden tot enzymactiviteit, wordt geïllustreerd met resultaten, verkregen in het erythrocyt-fibroblast en lymphocyt-lymphocyt en in het fibroblast-fibroblast celsysteem (artikel 8). Slechts één van beide vormen, namelijk overdracht van metabolieten, vindt plaats in het systeem met IMP-geladen erythrocyten en fibroblasten (artikel 9). Tenslotte zijn deze resultaten geplaatst tegen de achtergrond van de uit de literatuur bekende studies over metabole cooperatie. Die studies blijken zich vaak met verschillende vormen van metabole cooperatie bezig te houden, voornamelijk doordat niet dezelfde detectiemethoden worden gebruikt. Er is een voorlopige klassificering gegeven van de verschillende vormen van metabole cooperatie, waarover tot nu toe studies zijn gepublicieerd (artikel 10).

CURRICULUM VITAE

Martinus Petrus Uitendaal werd op 31 augustus 1949 te Driehuis-Velsen geboren als zoon van Mattheus Johannes Uitendaal en Margaretha Calvelage. In Driehuis bezocht hij de lagere school (St. Engelmundusschool) en vervolgens, van 1961 tot 1968, het Gymnasium Paulinum, alwaar hij het gymnasium β diploma behaalde. In september 1968 begon hij zijn studie scheikunde aan de Universiteit van Amsterdam. Het candidaatsexamen legde hij af in mei 1971, het doctoraalexamen (met paedagogische aantekening) in september 1974. De afstudeerrichtingen waren, als hoofdvak Biochemie, onder leiding van Prof.Dr. P.Borst, en als bijvak Experimentele Oncologie (op het researchlaboratorium van het Nederlands Kanker Instituut te Amsterdam), onder leiding van Prof.Dr. O.Mühlbock en Dr. J.H.M.Hilgers. Ook heeft hij drie maanden onderzoek gedaan aan hogedrukvloeistofchromatografie op het laboratorium van Prof.Dr. J.F.K.Huber. Vanaf september 1974 tot en met december 1977 is hij werkzaam geweest bij de biochemische groep van het Anthropogenetisch Instituut van de Katholieke Universiteit van Nijmegen (hoofd: Prof.Dr. S.J.Geerts). In het kader van zijn promotie onderzoek heeft hij de laatste jaren regelmatig op de afdeling Experimentele Cytogenetica (hoofd: Prof.Dr. P.Hösli) van het Institut Pateur te Parijs gewerkt.

STELLINGEN

behorende bij het proefschrift

Studies on Purine Metabolism in Cultured Human Fibroblasts

van

Martin P. Uitendaal

1. Adenosine deaminase activiteit in gekweekte fibroblasten wordt mede geregeld door nog onbekende kweekomstandigheden.

Dit proefschrift.

2. Aan de regulatoire rol van purine nucleoside phosphorylase in de purine stofwisseling wordt te weinig aandacht geschonken.

Dit proefschrift.

3. Toepassing van ultramicrochemische methoden opent nieuwe mogelijkheden voor experimenteel Anthropogenetisch onderzoek.

4. Tegenover de kosten, verbonden aan populatiegenetisch onderzoek naar draagsters van erfelijke afwijkingen door middel van haarwortelonderzoek, staan de preventief-geneeskundige baten.

5. De uitspraak van Pitts en Simms, dat volgens Kolodny nucleotiden niet van cel tot cel kunnen worden overgedragen, berust op onkritisch lezen.

J.D.Pitts and J.W.Simms, *Exp.Cell Res.*, 104 (1977) 153.
G.M.Kolodny, *Exp.Cell Res.*, 65 (1971) 313.
G.M.Kolodny, *J.Mol.Biol.*, 78 (1973) 197.

6. Aangezien Payne *et al.* bij incubaties van leukocyten met radioactief inosine verschillen van meer dan 50% in hypoxanthinegehalte in het medium tussen normale en Lesch-Nyhan cellen verwaarlozen, is hun conclusie over het bestaan van inosine kinase, die gebaseerd is op 1 à 2% incorporatie van label in IMP, voorbarig.

M.R.Payne, J.Dancis, P.H.Berman and M.E.Balis, *Exp.Cell Res.*, 59 (1970) 489.

7. Conclusies over het verband tussen abnormale adenosine deaminase activiteiten in bloedcellen en maligne afwijkingen zijn voorbarig zolang verschillende studies op dit gebied nog kwantitatief en soms ook kwalitatief inconsistente resultaten geven.

J.Zimmer, A.S.Khalifa and J.J.Lightbody, *Cancer Res.*, 35 (1975) 68.
J.F.Smyth and K.R.Harrap, *Br.J.Cancer*, 31 (1975) 544.
A.T.Huang, G.L.Logue and H.L.Engelbrecht, *Br.J.Haematol.*, 34 (1976) 631.
R.Tung, R.Silber, F.Quagliata, M.Conclyn, J.Gottesman and R.Hirschhorn, *J.Clin.Invest.*, 57 (1976) 756.

8. Wanneer aan de monospecificiteit van antisera hoge eisen worden gesteld, is het de moeite waard monoclonale antilichamen te bereiden door fusie van "non-secreting" myeloma cellen met miltcellen van geïmmuniseerde proefdieren en klonering van de hybride cellen.

G.Kohler and C.Milstein, *Eur J.Immunol.*, 6 (1976) 511.

9. De veronderstelling van Miyazaki en Katoh, dat hydroxyl-ionen isotachoforetisch geanalyseerd kunnen worden, berust op een foutieve interpretatie van hun resultaten.

H.Miyazaki and K.Katoh, *J.Chromatogr.*, 119 (1976) 369.

10. In zijn statistische berekeningen over de voor- en nadelen van het gebruik van saccharine in frisdranken met betrekking tot blaaskanker, onderschat Cohen de voordelen, doordat hij diabetici buiten beschouwing laat.

B.L.Cohen, *Nature*, 271 (1978) 492.

11. Het is onjuist te veronderstellen, dat de thans geheel in gevangenschap levende wereldpopulatie van Przewalskipaarden onder dierentuin-beheer voor de toekomst veilig is gesteld. Het verdient aanbeveling op korte termijn deze diersoort onder reservaat-omstandigheden in de natuur te herintroduceren.

12 Terroristische groeperingen die zich in oorlog met Nederland verklaren moeten zich realiseren, dat de Nederlandse rechtspraak in oorlogstijd de doodstraf kent.

13. Het feit, dat de enige eis die in Nederland aan Single Malt Scotch Whisky wordt gesteld een minimum alcoholpercentage van 35% is, doet een eenzijdige interesse van de Nederlandse overheid vermoeden.

14. Indien artsen hun witte jassen om hygienische redenen dragen, dienen ze die jassen voor het betreden van openbare ruimtes zoals personeelsrestaurants uit te trekken; als de jassen louter uit statusoverwegingen worden gedragen, verdient het aanbeveling ze nooit aan te trekken.

15. Winkeliers met de z.g. "fietstegels" voor hun zaak kunnen de service aan hun fietsende clientèle verhogen door regelmatig het afval uit de geulen van deze tegels te verwijderen.

16. Het feit, dat het concubinaat voor de bijstand wel, maar voor de WW en de belastingsdienst niet als band met financiele of andere verplichtingen geldt, wijst veeleer op een coöperatief-conservatieve houding van deze drie instanties samen, dan op een geëmancipeerde opvatting van de eerstgenoemde instantie.

Nijmegen, 14 juni 1978.

Martin P. Uitendaal