QUANTITATIVE RADIOCHEMICAL ENZYME ASSAYS IN SINGLE CELLS: PURINE PHOSPHORIBOSYL TRANSFERASE ACTIVITIES IN CULTURED FIBROBLASTS

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SUMMARY

An ultra-microchemical method using radioactive substrates has been developed for enzyme activity measurements at the single cell level. In order to demonstrate the possibilities of this radiochemical micro-assay, activity measurements of hypoxanthine-guanine phosphoribosyl transferase (HG-PRT) and of adenine phosphoribosyl transferase (A-PRT) in isolated human fibroblasts are described. There was a linear relationship between the number of cells incubated and the enzyme activities found. It was observed that the HG-PRT activity in single, skin derived, fibroblasts did not differ from that in amniotic fluid derived fibroblasts, thus providing a new, quantitative assay for rapid prenatal diagnosis in the Lesch-Nyhan syndrome.

Some problems in experimental cell biology and biochemistry can only be solved with micro- or ultra-microchemical methods. Ultra-microchemistry, which has been defined as measuring small amounts of substances or enzyme activities in volumes below 10 µl (1), has been predominantly developed by Lowry and associates (2). Due to the fact that Lowry's enzymatic cycling method is rather involved, it has been used only in a limited way by specialised workers in experimental research. On the other hand, microchemical methods have been succesfully introduced into practical clinical work by Mattenheimer (1). Less demanding ultra-microchemical methods might not only replace current micro-techniques for clinical work, but they would probably be used much more frequently for experimental investigations.

MATERIALS AND METHODS

A new relatively simple approach to ultra-microchemistry has been described by one of us (3). It is based on the development of the Plastic film Micro Cuvette (PMC). PMC's are very small incubation vessels with volumes of 1 µl or fractions of 1 µl. Immediately before use they are moulded in a parafilmstrip (Hösli, in preparation).After

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they have been filled with reaction mixture by means of a constriction pipette, they are sealed with a second parafilmstrip. The PMC technique has been introduced for the assay of enzyme activities with fluorogenic substrates (4). The present paper describes the use of this PMC technique with radioactive substrates.

For the cell culture studies the PMC technique is usually employed in conjunction with the Plastic Film Dish (PFD) (3,4). The PFD is comparable to a Petri dish, with an absolutely flat, transparent bottom. It consists essentially of a tripartite, commercially available holder (Tecnomara), which permits rapid mounting of a very thin, non-toxic plastic film. This combination makes it possible to assay activities of single, visually selected cells.

To demonstrate the PMC-based radio-micro assay, activity measurements of purine phosphoribosyl transferases in isolated human cells have been carried out. In man there are two different purine phosphoribosyl transferases: hypoxanthine-guanine phosphoribosyl transferase (HG-PRT; EC 2.4.2.8.) catalyses the conversion of the purine bases hypoxanthine and guanine to their corresponding mononucleosides, IMP and GMP respectively; adenine phosphoribosyl transferase (A-PRT; EC 2.4.2.7.) catalyses the conversion of adenine to AMP. Both reactions need phosphoribosylpyrophosphate (PRPP) as a co-substrate.

In outline the procedure is as follows: normal human diploid fibroblasts are cultured on the plastic film bottom of a PFD. After washing the cultures with physiological saline, the cells in the PFD are lyophilised in situ (3). Little plastic leaflets with counted numbers of cells are subsequently cut out of the bottom of the PFD. This cutting is done free-hand with a scalpel on a glass plate under a stereo microscope. The plastic leaflets with the counted numbers of cells are then transferred into PMC's containing 0.3 µl of incubation medium each. For the HG-PRT and A-PRT activity assays reported here the final concentrations of reagents in the reaction mixture were: 0.17 M Tris HCl buffer, (pH 7.4); 17 mM $MgCl_2$; 1.7 mM PRPP; 0.13 mM ^{14}C-labeled hypoxanthine, guanine or adenine (spec.act.57-59 mCi/mmole: radiochemical centre,Amersham). To avoid surface denaturation of the enzyme, which is particularly rapid in small incubation volumes, purified bovine serum albumin must be added (final concentration 0.05% w/v). To achieve sufficient amounts of product, the reaction mixture can be incubated at various temperatures for periods of up to several hours, depending on the predetermined stability of the enzyme. In the present studies incubation times of 2-8 hours were used at 37°. Reactions are terminated by pushing out the total contents of the PMC onto Whattman 3mm chromatography paperstrips (20x1 cm.). In the HG-PRT and A-PRT assays reported here, a sufficient separation of substrate and product was obtained after 30 minutes of descending chromatography, using 5% Na_2HPO_4 (pH 8.4) as a solvent. Standard solutions of unlabeled reference compounds were co-chromatographed and after inspection under U.V.light, purine base as well as nucleoside and nucleotide regions were cut out. Radioactivity was quantified by means of a Packard Tri Carb liquid scintillation counter.(counting fluid: toluene with PPO and POPOP). Counting efficiency was 68%.
With the Tri Carb counter a constant background of 30 $\pm$ 3 cpm has been found. If possible, we incubate for as many hours as is necessary to obtain a degree of product formation which leads to at least three times the background counts.

RESULTS AND DISCUSSION

In table 1 and figure 1 the means and ranges from 10 independent isolations of 1,2,4,8 and 16 cells are given. The range is due to the fact that the cells were isolated from asynchronously growing cell

Table 1.

Raw data from HG-PRT and A-PRT assays. For each number of cells, 10 individual isolations have been done and the corresponding means and ranges are given in counts per minute.

Enzyme assayed	number of cells	incubation time (hours)	net product formation (cpm) mean	range
HG-PRT with	1	8	182	116 - 243
hypoxanthine	2	6	273	188 - 355
	4	4	342	263 - 416
	8	3	457	386 - 612
	16	2	638	553 - 756
HG-PRT with	1	8	306	192 - 360
guanine	2	6	432	247 - 546
	4	4	517	412 - 657
	8	3	826	648 - 972
	16	2	1137	927 - 1284
A-PRT	1	8	318	177 - 382
	2	6	413	283 - 512
	4	4	536	386 - 618
	8	3	787	620 - 919
	16	2	1169	938 - 1326

cultures and therefore display cell cycle dependent enzyme activities. The means of 10 independent cell isolations show a good correlation between cell number and enzyme activity (fig.1 and fig.2). This is of practical importance for the rapid diagnosis of inborn errors of metabolism for instance. Figure 1 demonstrates the possibility of clear separation of the normal control and the HG-PRT deficient Lesch-Nyhan fibroblasts at the single cell level. If necessary it is even possible, in spite of the range of values introduced by the cell cycle, to make a diagnosis with a single cell isolation. In three strains of normal, skin derived fibroblasts and three strains of amniotic fluid derived fibroblasts, the HG-PRT activities were in the same range (fig.2), indicating that this quantitative micro-assay can be used directly for rapid prenatal diagnosis.

The principal advantage of this ultra-micro method is that there is a favourable signal/noise ratio: to increase the signal, long in-

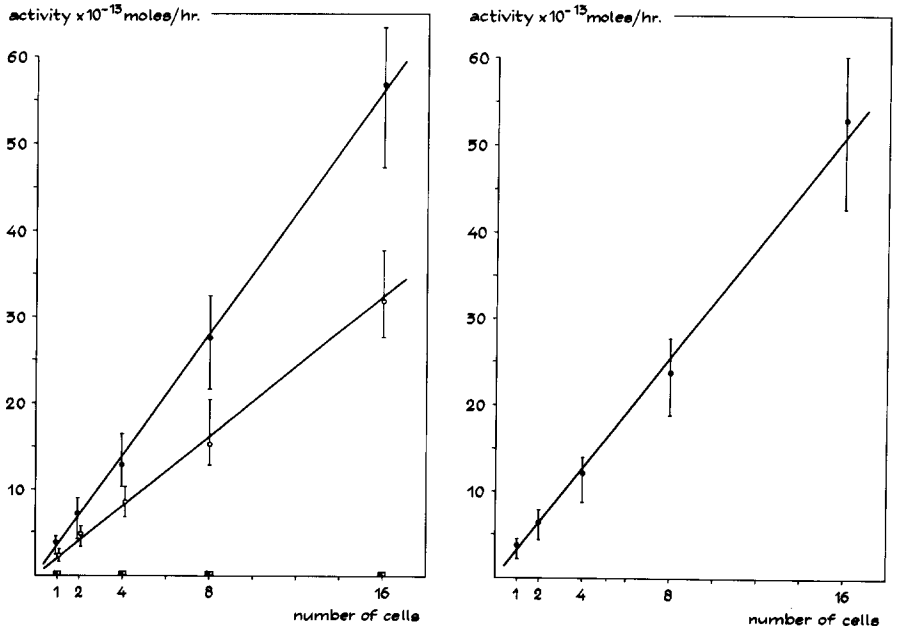

Figure 1.
HG-PRT (left) and A-PRT (right) activities in 10^{-13} moles per hour for different numbers of cells. For each point the means and ranges of ten independent isolations are indicated. In addition, HG-PRT activities of enzyme deficient diploid human Lesch-Nyhan cells are given. Open circles (left): HG-PRT with hypoxanthine as substrate in control cells; solid circles (left): HG-PRT with guanine as substrate in control cells; open squares (left): HG-PRT with hypoxanthine in Lesch-Nyhan cells; solid squares (left): HG-PRT with guanine in Lesch-Nyhan cells; solid circles (right) A-PRT in control cells.

cubation times are used; the noise (blank) is lowered by the small primary incubation volumes. The rationale for this is as follows: if radioactive substrates are not very pure, the noise will increase in proportion to the incubation volume unless all the contaminants can be removed. With conventional chromatografic procedures 5-10 µl samples can be handled at the utmost. If the primary reaction mixture exceeds this volume, aliquots have to be analysed, introducing sampling errors and decreasing the sensitivity of the assay.

The substrate and product radioactivity is always counted separately. The sum of these amounts of radioactivity should equal the radioactivity of the originally administered substrate. If this is not the case one has to look for side reaction products which will be found in different positions on the chromatografic paper. With our conditions (10 nmoles of unlabeled reference purines added to each 0.3 µl of reaction

mixture for co-chromatography) very reproducible separations were obtained. Extensive observations have shown that if handled in the right way, the PMC is always completely emptied onto the chromatografic paper.

In the present procedure the enzyme is brought into the PMC in a dry (lyophilised) state, thus avoiding technical errors due to micro-pipetting. Due to the complete emptying of the PMC and a good separation of substrate and product, these steps do not introduce detectable errors. If the method is used for assaying enzyme solutions, technical errors with a standard deviation of $\pm$ 6% due to micro-pipetting are introduced.

The characteristics of the PMC allow perfect temperature control of the enzyme reaction. For fluorogenic measurements, incubation is routinely done in a water bath. When working with hot substrates it is advisable, for health protection reasons, to work with a dry incubator.

With the set up described here enzymes which produce as little as 10^{-13} moles of product per hour can be assayed. The sensitivity of the method can be further increased by employing extremely long

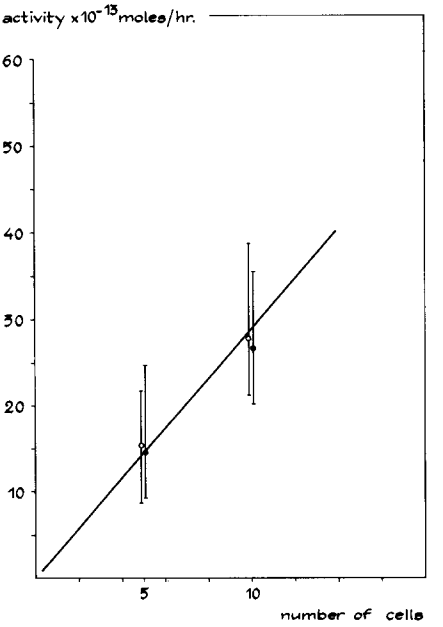

Figure 2.
HG-PRT measurements with guanine in 6 different fibroblastic cell strains: 3 were derived from normal skin and 3 from normal amniotic fluid. For each point 10 independent isolations were done. Solid circles indicate the results with the normal cell strains (mean and range); open circles indicate the amniotic fluid derived fibroblasts.

incubation and counting times. In addition, the sensitivity is considerably increased by using substrates with specific activities as high as possible (e.g. universaly labeled compounds). Current experiments show that it is then possible to measure enzyme activities even at the sub-cellular level.

As compared to fluorometric measurements, radiochemical assays offer two instrinsic advantages, namely the possibility of working with highly purified natural substrates and the ability to check possible side reactions. On the other hand,fluorogenic methods might make it possible to assay still lower enzyme activities; therefore, the radiochemical ultra-micro assay complements the fluorogenic technique. It seems highly probable that simple and reproducible methods of this kind will be used increasingly not only for experimental work, such as genetic complementation analysis(4,5,6)but also for practical clinical work (7,8,9).

It should be stressed that the method described is a general approach to ultra-micro chemistry. First, it is not restricted to the assay of enzyme activities, but can also be used for enzymatic estimation of a variety of substrates. Second, it is obvious that any currently known macro-enzyme assay can readily be adapted to the present ultra-micro-procedure, if substrates with relatively high specific activities are available.

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