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Antipyrine metabolism in cultured rat hepatocytes

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Antipyrine is metabolized to three main oxidative products by the cytochrome P-450 system. These products are 3-hydroxymethyl antipyrine (3OHA), 4-hydroxyantipyrine (4OHA) and norantipyrine (NORA). Antipyrine has, therefore, been proposed as a probe of drug oxidation in animals and man [1]. Selective effects on the formation of these metabolites have been shown with inducers such as phenobarbital and β-naphthoflavone in rats [1–3] and in man [4] and by the use of inhibitors [5]. Several groups have investigated the effects of induction upon antipyrine metabolite pattern in rat urine [1, 3, 5, 6]. All of these authors found selective changes in metabolite profile as a consequence of induction suggesting that several isozymes of cytochrome P-450 metabolize antipyrine, although significant variability in urinary metabolite patterns was found between groups. Rhodes and Houston, [3] showed that induction with phenobarbital increased the *in vivo* formation rate constant of 4OHA by 200%. As a consequence 4OHA was the major urinary metabolite of antipyrine in rats pretreated with phenobarbital.

The use of isolated and cultured hepatocytes to generate metabolite profiles of novel drugs has recently been proposed [7]. However, the partial loss of differentiated function of hepatocytes in culture is well-documented [8]. In particular, losses in cytochrome P-450 levels are commonly observed within the first day of culture [9] although these losses may be modified by the addition of ligands to the culture medium [10]. Little information is available on the metabolism of drugs and xenobiotics by hepatocytes maintained in culture. Thus, the aim of this study is to investigate the utility of antipyrine metabolite formation as a probe of changes in cytochrome P-450 content of hepatocytes maintained in culture.

Materials and methods

Chemicals. The chemicals and reagents used in this study were of the highest quality generally available. 3-hydroxymethyl-antipyrine, 4-hydroxyantipyrine and norantipyrine were synthesized by the Department of Synthetic and Isotope Chemistry, SK&F.

Animals. Rats (Wistar, male) were obtained in the range 180–200 g from the SK&F colony and were housed on grade 6 greenwood granules in polypropylene cages. Free access to tap water and PRD pellets (Labsure, Poole, Dorset, U.K.) was provided.

Induction of rat liver microsomal activity. Rats were administered phenobarbital for 7 days in the drinking water ($1\ mg \cdot ml^{-1}$) [11]. β-Naphthoflavone (βNF) was administered on two consecutive days ($80\ mg \cdot kg^{-1}$ in corn oil, i.p.) and hepatocytes were prepared 24 hr after the last dose.

Preparation of hepatocytes. Hepatocytes were prepared by the technique of Strom *et al.* [12], and were then counted in a haemocytometer in the presence of 0.04% trypan blue. Yields of 30–40 $\times$ 10^6 cells were commonly achieved from each lobe with a viability in excess of 90%.

Hepatocytes were quickly diluted into culture medium consisting of Williams Medium E containing L-glutamine (4 mM), penicillin ($100\ IU\ ml^{-1}$), streptomycin ($100\ \mu g \cdot ml^{-1}$), neomycin ($100\ \mu g \cdot ml^{-1}$), insulin ($0.02\ IU\ ml^{-1}$) and newborn calf serum (10% v/v). The cell suspension was then seeded on to 35 mm wells (Sterilin) which had been coated with soluble collagen. The culture dishes were then introduced into a 37° incubator (T. R. Heraeus) containing 5% CO_2 in a water saturated atmosphere. Cell attachment could be detected after about one hour in culture. When cells were maintained in culture the medium was renewed every 24 hr.

Measurement of cytochrome P-450. Cell culture medium was removed and the cell monolayer washed with saline. A solubilising buffer was then added to each culture plate (1 ml to a 35 mm well). The buffer was exactly as described by Warner *et al.* [13] except that Renex 690 (Atlas Chemicals, Surrey, U.K.) was substituted for Emulgen 911. After 5 min at room temperature the cell monolayer was scraped into the buffer and the contents of two wells combined.

The buffer was then centrifuged (1000 *g*, 5 min, 21°, Sorvall RT-6000) to remove debris. The buffer, containing the solubilised cytochrome P-450, was then transferred to a clean tube. Cytochrome P-450 was measured by the method of Omura and Sato, [14] utilising a Hewlett–Packard 8450A diode array spectrophotometer.

Measurement of enzyme activities. 7-Ethoxycoumarin O-deethylation (ECOD) was measured by the formation of

7-hydroxycoumarin [15]. 7-Ethoxycoumarin was dissolved directly in Williams Medium E and incubations were performed in a CO_2 incubator at 37°. The reaction was generally stopped after two hours by removing the medium from the cell monolayer. A mixture of sulphatase (0.05 U, type IV, Sigma) and β-glucuronidase (25 U, type VII, Sigma) was added to the medium from each well (2 ml) and incubated at 37° for 30 min. Hydrolysis was complete within 2–3 min with this system · 1 ml of medium was then taken for measurement of 7-hydroxycoumarin content in a Perkin–Elmer MPF 44A fluorescence spectrophotometer.

The cell monolayer was washed with saline and dissolved in 1.0 M NaOH. Protein was determined by the method of Lowry *et al.* [16].

Hydrolysis and assay of antipyrine and antipyrine metabolites. Samples were hydrolysed with a mixture of sulphatase (0.05 U, type VI, Sigma) and β-glucuronidase (25 U, type VII, Sigma). These enzymes were added to the medium from each well after the addition of sodium metabisulphite (1 mg · ml^{-1}) as anti-oxidant. Samples were then stored at −20° in a stoppered 10 ml polypropylene tube prior to analysis. Phenacetin was added as internal standard.

Antipyrine and its metabolites were separated by HPLC and quantified by 254 nm u.v. detection using an isocratic system, 250 × 4.6 mm Waters C_{18} μBondapak column held at 40°. The solvent system consisted of 0.02 M phosphate buffer containing 0.02 M ammonium acetate, 10% acetonitrile and 10% methanol adjusted to pH 7.1. The flow rate was 2 ml · min^{-1}. Under these conditions the retention times for antipyrine and its metabolites were: 3OHA (3.3 min), NORA (4.4 min), antipyrine (6.2 min), 4OHA (8.0 min) and phenacetin (11.5 min).

Analysis of data. The half-life of antipyrine in the culture medium was calculated by regression analysis of semi-logarithmic plots. Rates of formation of metabolites of antipyrine were calculated from the linear portion of the appearance curves. Intrinsic clearance (Cl_{int}) was calculated from the half-life estimate using the relationship

$$Cl_{int} = \frac{\text{volume of medium} \times 0.693}{\text{Half-life}}$$

Results and discussion

(i) Hydrolysis of antipyrine metabolites. The primary metabolites of antipyrine are extensively conjugated. Hydrolysis was therefore performed enzymatically with sulphatase and β-glucuronidase. The hydrolysis of antipyrine metabolites was judged to be complete within 10 min under the experimental conditions described. No subsequent change in metabolite profile was then observed over the next 50 min. A 30-min incubation was therefore used in subsequent experiments.

(ii) Antipyrine disappearance kinetics. The disappearance kinetics of antipyrine were studied in hepatocytes prepared from control rats and also from rats treated with phenobarbital. These data are consistent with the metabolism of antipyrine being a first-order process throughout the entire incubation period in all preparations. The intrinsic clearance (Cl_{int}) for control cells was 0.021–0.034 ml · hr^{-1} · mg $protein^{-1}$ in two separate preparations. In contrast, cells produced from two rats treated with phenobarbital exhibited Cl_{int} values of 0.082–0.099 ml · hr^{-1} · mg $protein^{-1}$. These data were generated at an initial target concentration of 30 μM.

(iii) Time-course of metabolite formation. Linearity of metabolite formation with time was investigated throughout a 4 hr incubation period in hepatocytes from rats treated with phenobarbital. The linearity was measured in a hepatocyte preparation maintained in culture for 2 hr, 24 hr and 48 hr (Fig. 1). The rate of formation of 4OHA was approximately linear over 4 hr incubation. In the case of 3OHA linearity of metabolite formation was lost after 2 hr incubation although this trend was much less noticeable at 48 hr culture than at 2 hr culture. This non-linearity was even more marked with NORA. At 2 hr of culture, NORA accumulated up to maximal levels at 3 hr of incubation but by 4 hr of incubation the levels of NORA in the culture medium had started to decline. Non-linear accumulation of NORA was also observed in hepatocytes cultured for 24 and 48 hr. Thus, the elimination of antipyrine through metabolism remained first-order throughout a 5 hr incubation period whilst the accumulation of metabolites in the culture medium demonstrated a distinct non-linearity, especially in the case of NORA and to a more limited extent in the case of 3OHA. Although the mechanism of non-linearity was not pursued it would seem likely that further metabolism of NORA and 3OHA, other than conjugation reactions, occurred in this system [17].

(iv) The effect of inhibitors on metabolite formation. The inhibition of drug metabolism by chloramphenicol, a selective inhibitor of certain cytochrome P-450 isoenzymes [18] was investigated with the model substrate ethoxycoumarin in hepatocytes from control rats and from rats induced with phenobarbital and β-NF. Pretreatment of hepatocytes with chloramphenicol for two hours had no effect on ECOD activity in hepatocytes from β-NF-treated rats, caused a modest inhibition of activity in control hepatocytes and a marked inhibition of activity in hepatocytes prepared from phenobarbital-treated rats (Fig. 2a). These observations not only demonstrate the selectivity of chloramphenicol inhibition but also demonstrate that chloramphenicol pretreatment had no detectable indirect effects on drug metabolism such as on the capacity of cellular NADPH-generating systems [19].

The effects of preincubation with chloramphenicol on antipyrine metabolite formation were investigated in hepatocytes from phenobarbital-treated rats (Fig. 2b). The formation of all metabolites was reduced after pretreatment with chloramphenicol. The kinetics of loss of activity was complex in each case, with a very rapid initial loss of activity followed by a slower decline. Thus, the extent of inhibition of each metabolite pathway by chloramphenicol treatment could be ranked NORA > 4OHA > 3OHA. This would suggest that more than one form of cytochrome P-450 must be responsible for the metabolism of antipyrine in these hepatocyte preparations.

(v) The effect of culture time on antipyrine metabolite formation. Table 1 shows the effect of culture time on the cytochrome P-450 content of hepatocytes from phenobarbital-treated rats. In the absence of added metyrapone the cytochrome P-450 content decreased markedly between 2 hr and 24 hr but not between 24 hr and 48 hr. In the presence of 0.5 mM metyrapone the loss in cytochrome P-450 by 24 hr was not statistically significant. By 48 hr, however, the loss in total cytochrome P-450 had decreased significantly ($P < 0.05$), compared with values obtained at 2 hr.

Table 1. Effect of duration of culture on total cytochrome P-450 levels in hepatocytes from phenobarbital-treated rats

Duration	Cytochrome P-450 (mean ± SD) (pmol · mg $protein^{-1}$) WME	Metyrapone 0.5 mM)
2 hr	504 ± 22 (N = 4)	409 ± 71 (N = 6)
24 hr	*177 ± 29 (N = 4)	352 ± 37 (N = 4)
48 hr	*149 ± 12 (N = 2)	†317 ± 24 (N = 3)

* $P < 0.01$ compared to metyrapone value at 2 hr.

† $P < 0.05$ compared to Williams medium (WME) value at 2 hr.

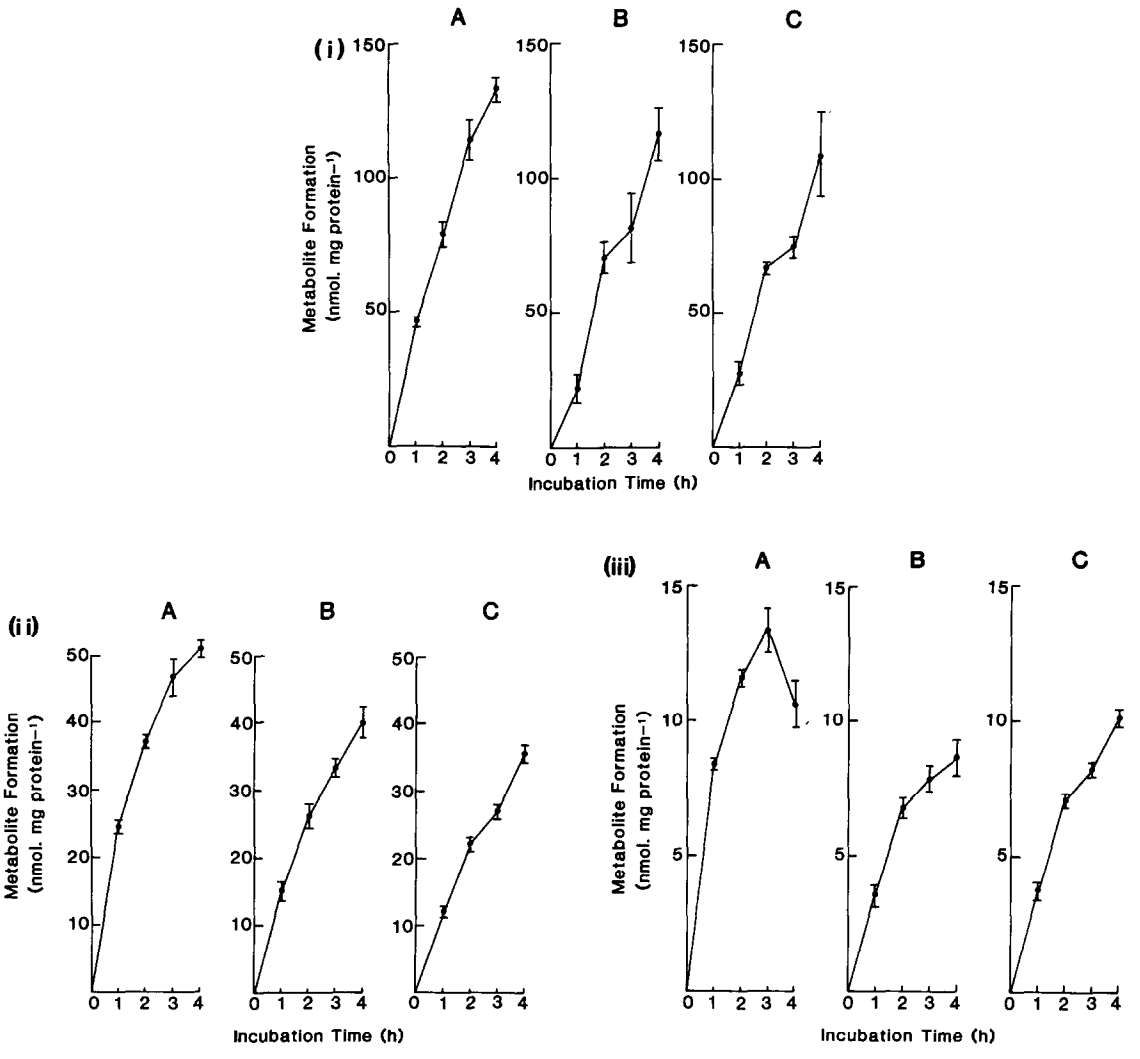

Fig. 1. The time-course of 4OHA, 3OHA and NORA formation by hepatocytes prepared from a phenobarbital-treated rat. Hepatocytes were prepared and introduced into culture as described in Methods. After 2 hr (A), 24 hr (B) or 48 hr (C) in culture, medium was replaced by 1 ml of incubation medium containing 5 mM antipyrine. After the appropriate time-interval medium was removed and the formation of 4OHA (i), 3OHA (ii) and NORA (iii) analysed by HPLC. 1 ml of 1.0 M NaOH was added to each well and protein determined [17]. Mean and standard deviation of three replicates are presented.

In control cultures, all three metabolite pathways showed a statistically significant decline of rate of formation of metabolite at 24 hr compared to two hours of culture ($P < 0.01$) but no further decline between 24 hr and 48 hr (Table 2). The extent of loss of activity at 24 hr could be ranked NORA > 3OHA > 4OHA (53%, 37% and 34% decline respectively). In contrast hepatocytes cultured in the presence of metyrapone exhibited a modified pattern of loss of activity. At 24 hr of culture statistically significant losses in activity were observed with 4OHA and NORA but not 3OHA. Thus, the loss of activity could be ranked NORA > 4OHA > 3OHA (53%, 21% and 10% decline respectively). In the culture period between 24 hr and 48 hr significant losses of the 3OHA activity were then observed although the other two activities remained stable in the cultures treated with metyrapone. Thus, maintenance of hepatocytes in culture for periods of 1 or 2 days resulted in a consistent loss of all three major pathways of metabolism monitored in this study. Therefore, the metabolic profile of antipyrine seen in these cells changed quantitatively but remained qualitatively similar over this period of culture. Similar observations have been made with the metabolism of pindolol and fluperlapine in cultured hepatocytes [9]. Inclusion of metyrapone in the culture medium helped to stabilize both the total cytochrome P-450 levels and the rates of total metabolite formation. However, the effects of metyrapone on antipyrine metabolism were selective. The formation of 4OHA was well-maintained even after 2 days in culture but losses of NORA formation appeared to be accelerated in the presence of metyrapone.

In conclusion the metabolism of antipyrine in cultured hepatocytes involves several cytochrome P-450 isozymes. Therefore, changes in isozyme composition through induction or suicidal inhibition with chloramphenicol were reflected in selective changes in the formation of individual metabolites. This technique, therefore, offers valuable information on the composition and function of cytochrome P-450 isozymes in the cultured hepatocyte system as well as other models.

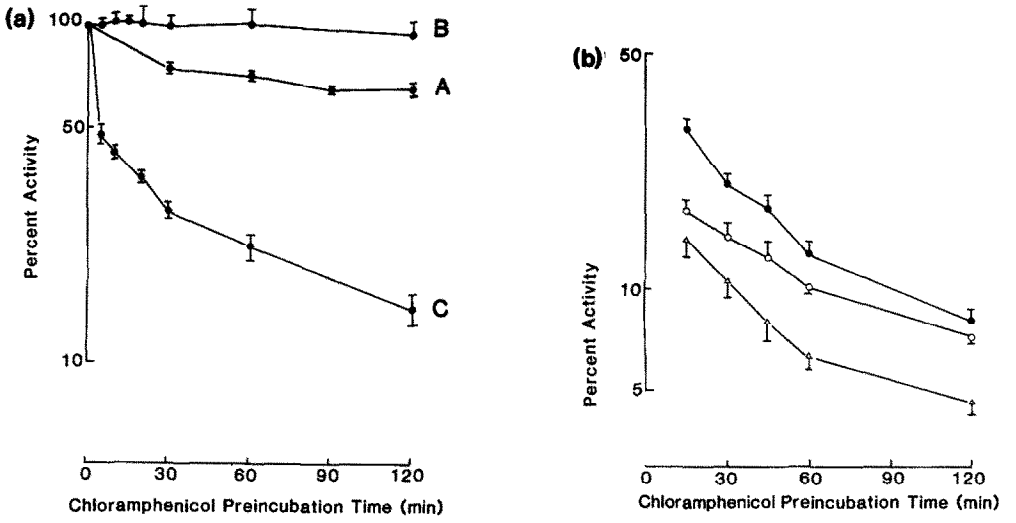

Fig. 2. The effect of various times of chloramphenicol pretreatment *in vitro* on ECOD (a) and antipyrine (b) metabolism in hepatocytes. Hepatocytes were prepared and introduced into culture as described in Methods. Cells were then treated with fresh incubation medium containing 1 mM chloramphenicol for various periods of time. The incubation medium containing chloramphenicol was then removed, the monolayer washed, and incubation medium containing ethoxycoumarin (610 μM) or antipyrine (5 mM) added. The formation of metabolite was then determined after a 2-hr incubation period. Initial activity for control cells (A) was 13.5 ± 1.4 (N = 6) nmol · mg protein^{-1} · hr^{-1} at 610 μM ethoxycoumarin. Initial activities for cells from phenobarbital (C) or β-NF-(B) treated rats were 64.9 ± 5.3 (N = 6) and 131.6 ± 1.6 (N = 5) nmol · mg protein^{-1} · hr^{-1} respectively at 610 μM ethoxycoumarin. The formation of 3OHA (closed circle), 4OHA (open circle) and NORA (open triangle) is also presented. The data are presented as percentage mean ± standard deviations of initial activity without pretreatment with chloramphenicol (N = 6 replicate plates).

Table 2. The effect of duration of culture on antypyrine metabolite formation in hepatocytes from phenobarbital-treated rats

	Percentage activity					
	Control			Metyrapone (0.5 mM)		
Day	3OHA	4OHA	NORA	3OHA	4OHA	NORA
1 (24 hr)	*63.1 ±7.4	*65.8 ±19.2	*47.4 ±3.7	89.6 ±7.0	*79.0 ±11.1	*47.4 ±5.9
2 (48 hr)	*54.3 ±2.7	*69.7 ±9.0	*50.2 ±4.3	*57.6 ±1.3	*80.7 ±7.1	*36.4 ±4.8

Hepatocytes were isolated and introduced into culture as described in Materials and methods except that metyrapone (0.5 mM) was included in the culture medium where appropriate. Rates of formation of metabolites in cells cultured for 2 hr were 22.3 ± 3.4, 43.8 ± 4.0 and 7.2 ± 1.6 nmol · hr^{-1} · mg protein^{-1} for 3OHA, 4OHA and NORA respectively in the absence of metyrapone and 17.3 ± 1.8, 37.5 ± 1.5 and 5.3 ± 1.0 nmol · hr^{-1} · mg · protein^{-1} for 3OHA, 4OHA and NORA respectively in cells cultured with metyrapone. *$P < 0.01$ compared to 2 hr activity.

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The prolongation of the survival times of mice implanted with TLX5 cells by treatment with methotrexate encapsulated in erythrocytes

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By using gentle loading techniques it is possible to encapsulate drugs into erythrocytes which when resealed will survive upon return to the circulation [1]. Possible applications of this technique to cancer therapy reviewed include the encapsulation of enzymes such as asparaginase to remove tumour-promoting amino acids or the encapsulation of lower molecular weight drugs which after encapsulation may diffuse slowly from the cell into the circulation [2]. A possible advantage of this type of slow-release system is that until the drug is released into the circulation it is not subjected to inactivation by metabolism. This may prolong the therapeutic efficacy of the drug over a much longer period than when the drug is administered systemically, allowing less frequent dosage.

In this communication we have encapsulated methotrexate (MTX) into erythrocytes and examined the effect of the encapsulated preparation on the survival time of CBA/CA mice implanted with TLX5 tumour cells. The rate of release of methotrexate from erythrocytes was also studied *in vivo* and *in vitro*.

MTX was encapsulated in CBA/CA male mouse erythrocytes by a technique based on pre-swelling erythrocytes in hypotonic solutions [3]. In this method blood was obtained by cardiac puncture from CBA/CA male mice by a heparinized syringe. The pooled blood from several animals was centrifuged at 600 *g* for 7 min. The plasma and white cells were discarded. A 1 ml portion of the packed erythrocytes was transferred to a plastic centrifuge tube and 4 ml of a modified K^+-reversed Hank's solution was added with gentle mixing by inversion (KCl 10.18 g/l, KH_2PO_4 0.1 g/l, $NaHCO_3$ 1.273 g/l, NaCl 0.316 g/l, $Na_2HPO_42H_2O$ 0.1 g/l and glucose 2.0 g/l). The Hank's solution had been diluted with water to adjust its final tonicity to 0.67 that of the cells. The cell suspension was centrifuged at 600 *g* for 5 min and the supernatant removed leaving a pellet of "pre-swollen" erythrocytes. A separate portion of packed erythrocytes was lysed by the addition of an equal volume of water and 0.2 ml of this lysate was layered on top of the cells. Meanwhile MTX was dissolved in 0.05 M $NaHCO_3$ to give a final concentration of 8 mg/ml. The MTX used contained 7 μCi of ^{3}H-MTX as a tracer (Amersham International plc, Amersham, U.K.). The purity of the radiolabelled material was confirmed by us as in excess of 96% when we examined the material by paper chromatography using *n*-butanol saturated with 2N ammonia as developing solvent followed by scanning the dried chromatogram using a 4II windowless gas flow radiochromatogram scanner (Tracerlab Instruments). Radiolabelled MTX was used as quickly as possible after receipt in order to avoid stability problems.

Two 0.1 ml portions of the MTX solution was layered on top of the swollen erythrocyte suspension and the tube contents were mixed by gentle inversion. The tube was centrifuged at 600 *g* for 5 min. At this stage the supernatant layer was observed to have darkened but was clearly differentiated from the layer of packed cells. Accordingly a further 0.05 ml of MTX solution was layered on to the supernatant and mixed with the cells by gently inverting the tube. The tube was centrifuged at 600 *g* for 5 min and the supernatant examined. It was observed that the supernatant layer had darkened further and that a small number of erythrocyte ghosts were present at the interface between the supernatant and the packed cells. These "ghosts" were probably due to the lysis of older cells which have greater osmotic fragility. Since the visual examination indicated that the cells were approaching the point of lysis [4] they were resealed by adding the calculated amount of 10 times hypertonic Hank's balanced salt solution (HBSS) to restore isotonicity to the cells. The cells were centrifuged at 600 *g* for 7 min and the supernatant discarded. The cells were washed twice with normal HBSS to remove excess MTX. As a control the same amount of MTX was incubated