enhanced cholinergic activity by malathion may induce the release of certain humoral factors or somatostatin which might be involved in the mobilization of glycogen leading to hyperglycaemia [25–27]. The possible involvement of a cholinergic mechanism is suggested by the finding that atropine, a known blocker of cholinergic activity, abolished the hyperglycaemia and reduction in the level of glycogen in various brain structures of malathion-treated rats (Table 1).

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Industrial Toxicology Research Centre
Mahatma Gandhi Marg
Lucknow, U.P., India

MOHD A. MATIN*
ROSHAN A. SIDDIQUI

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* Address all correspondence to: Dr. M. A. Matin, Industrial Toxicology Research Centre, P.B. 80, Lucknow, India.

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Filter trapping of $^{14}CO_2$: a simple and quantitative method for studying cell metabolism in hepatocyte monolayers

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Quantitative analysis of $^{14}CO_2$, appearing in the exhaled air after administration of specifically ^{14}C-labelled aminopyrine has been extensively used as a noninvasive method to study oxidative xenobiotic metabolism *in vivo* both in animals [1–4] and in man [5–8]. Although an *in vitro* application of this approach was shown to be feasible in hepatocyte suspensions [9, 10], its widespread use has been hampered owing to the lack of a simple, yet quantitative CO_2 collection system. Since, compared to hepatocyte suspensions, monolayer cultures offer several advantages (e.g., recovery from isolation procedure, cell to cell contact, prolonged viability), we have developed and tested a CO_2 collection system in chick embryo hepatocyte monolayers. This paper focuses on the use of the system for metabolic studies with ^{14}C-labelled aminopyrine; however, it is basically applicable to the study of a wider variety of compounds, which contain *N*-methyl moieties and yield C_1 fragments during biotransformation.

Materials and methods

Preparation of hepatocyte monolayers. The livers of 16 days old chick embryos (crossbred shaver strain) were prepared according to the method of Granick *et al.* [11] as modified by Meyer *et al.* [12]. The collagenase used was purchased from Millipore Corp., (Freehold, NJ) the Hanks BSS without Ca^{2+} and Mg^{2+} from Gibco-Biocult (Paisley, U.K.). The hepatocytes were plated at a density of 2.4×10^5 cells/cm^2 in Falcon T25 culture flasks (Becton-Dickinson,

Cockeysville, MA), using 3 ml Williams E medium (Flow Lab, Irvine, U.K.) supplemented with 300 μl 10% fetal calf serum, 50 μg/ml gentamycin and 1 μmole/ml L-glutamine. After 5 hr incubation at 37.5° in 95% air and 5% CO_2, the medium was replaced by new, serum-free Williams E medium containing 1 μg/ml insulin. Subsequently, the serum-free medium was exchanged every 24 hr. All studies were carried out in monolayers 48 hr after plating. Protein content of the monolayers was determined at the end of each experiment using the method of Lowry *et al.* [13].

$^{14}CO_2$ *trapping system.* The newly designed trapping system for $^{14}CO_2$ consists of a 2.4 cm Whatman GF/A microfilter paper, which is soaked with 200 μl 0.5 M NaOH and placed in a small plastic center well (purchased from Kontes Glass Company, Vineland, NJ). Through a resealable opening of the screw-cap, the plastic centre well is suspended in the culture flask, and the screw-cap tightly closed (Fig. 1). After different time intervals of incubation at 37°, the filter may be removed, transferred to a counting vial containing 5 ml Lumagel (Lumac Systems, Meise, Belgium) and counted in a scintillation counter.

To assess the efficiency of the $^{14}CO_2$ trapping system, three tracer doses (0.003, 0.03 and 0.3 μCi) of [^{14}C]sodium bicarbonate (10 mCi/nmole; T.C.N. Irvine, CA) were added to 12 monolayers for each period of incubation, the filters removed at hourly intervals and counted. The cumulative $^{14}CO_2$ yield after 1 hr of incubation was 97.5 ± S.D. 5% of the dose of radioactivity added, suggesting that filter trapping of newly formed $^{14}CO_2$ was virtually complete. Since the values at 2,3,4 and 5 hr remained at 94 ± 4, 93 ± 4, 98 ± 8 and 97 ± 5%, respectively, it may be inferred that potential loss of $^{14}CO_2$ from culture flasks, if it occurred, was negligible.

N-*Demethylation of aminopyrine.* In a first set of experiments, aminopyrine demethylation was measured by adding tracer doses (0.1 μCi per flask) of [^{14}C-dimethyl]aminopyrine (25 mCi/mmole; The Radiochemical Center, Amersham, U.K.) dissolved in 0·9% saline (pH 7.3) to the monolayers. $^{14}CO_2$ resulting from hepatocellular metabolism was captured on a filter (as described above) during incubation periods of 1–6 hr, and radioactivity counted. For enzyme kinetic studies of aminopyrine demethylation, the procedure was identical, except that unlabelled aminopyrine (concn range 0.05–25.6 mM) was mixed with 0.1 μCi of the labelled compound prior to addition to the monolayers. Cumulative $^{14}CO_2$ yield was then measured during a period of 3 hr.

In a second set of experiments, the effects of enzyme induction and inhibition, and of cell damage due to carbon tetrachloride on aminopyrine demethylation were assessed. Enzyme induction and inhibition were achieved according to the procedure described by Poland *et al.* [14], using 400 μg/flask of phenobarbital and 4 μg/flask testosterone, respectively. Cumulative $^{14}CO_2$ yield was measured up to 6 hr. For production of cell damage, monolayers were exposed during 5 hr to different doses (0.02, 0.2 and 2 μl/ml medium) of carbon tetrachloride (E. Merck, Darmstadt, West Germany). Prior to addition of aminopyrine, residual CCl_4 was washed out by medium exchange.

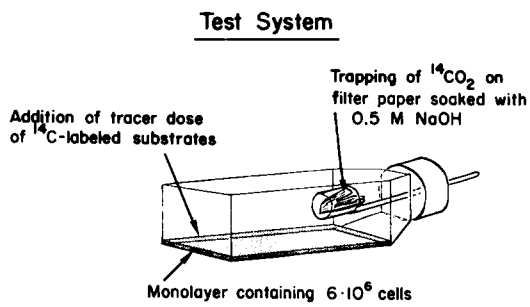

Fig. 1. Through a resealable opening of the screw cap, a plastic centre well (containing the microfilter paper) is suspended in the culture flask.

Results

Time dependence of $^{14}CO_2$ yield following addition of tracer doses of [^{14}C]aminopyrine in control cultures, and following enzyme induction or inhibition is shown in Fig. 2. It is evident that during the 6 hr of observation, $^{14}CO_2$ yield was linear with time. Compared to controls, demethylation was increased and decreased, respectively, in monolayers induced with phenobarbital or inhibited with testosterone. After induction, the slope of the regression line of time-dependent $^{14}CO_2$ yield was 257 dpm/mg protein/hr, that after inhibition 28 dpm/mg protein/hr, both slopes being significantly ($P < 0.01$) different from control (54 dpm/mg protein/hr). This difference in cumulative $^{14}CO_2$ activity achieved statistical significance already after 3 hr of incubation, suggesting that single point measurement following a relatively short period of incubation is sufficient to discern the induced changes.

Based on the apparent reliability of this simple $^{14}CO_2$ trapping system, enzyme kinetic studies of aminopyrine demethylation were performed in control monolayers and cultures induced with phenobarbital, incubated during 3 hr in the presence of 0.05–25.6 mM of the compound. The Lineweaver–Burk plot of the rate of *N*-demethylation as measured by $^{14}CO_2$ formation was non-linear in control cultures, yielding a low apparent K_m of 0.03 mM and a high apparent K_m of 1.8 mM, with corresponding V_{max} values of 3.1 pmoles/mg protein/min and 26.5 pmoles/mg protein/min, respectively. Non-linear kinetics were also observed in the cultures pretreated with phenobarbital; as expected, induction resulted in a predominant change of the maximal velocities, V_{max} increasing to 5.0 for the 'high-affinity' and to 36.5 for the 'low affinity' enzyme.

As depicted in Fig. 3, the simple approach with the $^{14}CO_2$ trapping system may be suitable for assessing cell damage due to chemicals such as carbon tetrachloride. Evidently, increasing doses of CCl_4 resulted in a marked depression of aminopyrine demethylation. Thus, cumulative $^{14}CO_2$ yield, collected during 3 hr after addition of labelled aminopyrine, was reduced to 66, 21 and 8% of control with doses of 0.06, 0.6 and 6 μl CCl_4 per flask, respectively. Figure 3 may be viewed as a dose–response relationship of the toxic effects of CCl_4 on microsomal cell function.

Discussion

The aim of this study was to define the characteristics and describe the usefulness of the newly developed $^{14}CO_2$ filter trapping system. If this system was to be employed for studies of drug metabolism or drug toxicity in hepatocyte monolayers, it had to satisfy the following criteria; simplicity, allowing simultaneous application to numerous culture flasks; non-invasiveness, permitting studies without disturbing cell integrity of the monolayers; high sensitivity, allowing quantification of a metabolic end product ($^{14}CO_2$) at short time intervals (hours).

We believe that our approach using a plastic well-mounted filter paper soaked with NaOH for trapping of $^{14}CO_2$ fulfils these criteria. It may be easily handled and is simultaneously applicable to large numbers of cultures. Since the procedure is non-invasive, it may be reasonably assumed that metabolism in monolayers is studied under optimal conditions. Finally, as demonstrated using ^{14}C-labelled bicarbonate, recovery of $^{14}CO_2$ up to 5 hr collection time is equal to or greater than 93%.

Consequently, following the addition of small amounts (usually 0.1 μCi per flask) of the specifically labelled substrate, counting yield is sufficient to limit the collection time for $^{14}CO_2$ to a few hours. The short collection period is extremely practical, when the procedure is used for

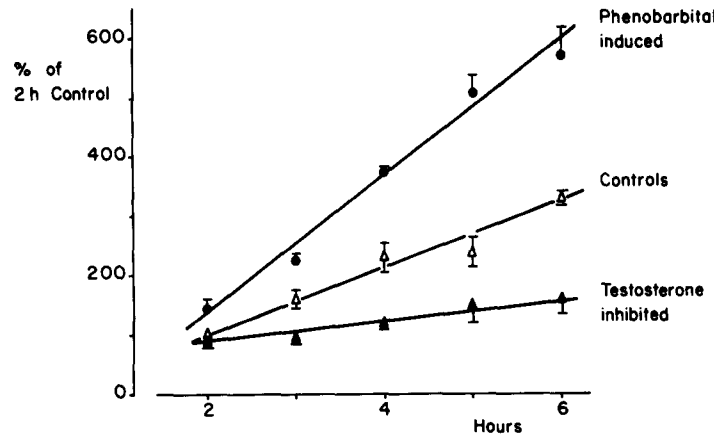

Fig. 2. Aminopyrine *N*-demethylation in control hepatocyte monolayers (△), and monolayers induced with phenobarbital (●) or inhibited with testosterone (▲). In all situations, $^{14}CO_2$ yield from tracer doses (0.1 μCi per flask) of labelled aminopyrine is linear with time. The slopes of the regression lines following enzyme induction or inhibition are significantly ($P < 0.01$) different from control. Each point represents the mean ± S.D. of 5 monolayers.

assessing the viability of monolayers. Thus in testing selected cultures using tracer doses of [^{14}C]aminopyrine prior to performing drug metabolism or drug toxicity studies, we have been able to minimize interbatch variation in our results, since batches with a $^{14}CO_2$ yield of less than 10^3 dpm/mg protein in 3 hr are eliminated. On the other hand, the limited collection time may also be important in relation to cell function, since it might be argued that CO_2-trapping changes the milieu of the hepatocytes. Although measurement of cytochrome P-450 [15] in monolayers exposed to 6 hr of CO_2 filter-trapping has yielded values (0.099 ± S.D. 0.024 nmoles/mg protein; $n = 6$) comparable to control cultures (0.106 ± 0.043), the possibility of interference due to prolonged depletion of CO_2 cannot be ruled out.

The usefulness of the system was demonstrated by studying the metabolism of aminopyrine, a classical substrate for microsomal demethylation [16], both with tracer amounts and with saturating doses. The finding that $^{14}CO_2$ yield is linear with time suggests that our procedure is capable of estimating the rate limiting step in the metabolic event leading to CO_2 formation. This contention is further supported by the differences in slopes observed between $^{14}CO_2$ yield from cultures induced with phenobarbital and inhibited with testosterone, the former finding being in agreement with the results of Poland and Kappas, who demonstrated increased *N*-demethylase activity in chick-embryo monolayer homogenates following phenobarbital pretreatment [14].

In addition, our kinetic studies pointed to 'low affinity' and 'high affinity' enzymes of comparable activity to those found in microsomal preparation [14].

Finally, the potential usefulness of our system to the investigation of toxic cell injury was also demonstrated. Experiments using different doses of the classical hepatotoxin carbon tetrachloride served to show that a direct dose-response relationship may be established using [^{14}C]aminopyrine demethylation. This result was to be expected, since previous studies clearly implicated the microsomal drug metabolizing system as a target of CCl_4 toxicity [17].

In summary, a $^{14}CO_2$ trapping system consisting of a 2.4 cm Whatman GF/A microfilter paper soaked with NaOH and suspended with a plastic centre well in the culture flask, was tested in chick embryo hepatocyte monolayers. $^{14}CO_2$ recovery up to 5 hr following addition of ^{14}C-labelled bicarbonate was equal to or greater than 93% of the dose. $^{14}CO_2$ yield from tracer doses of

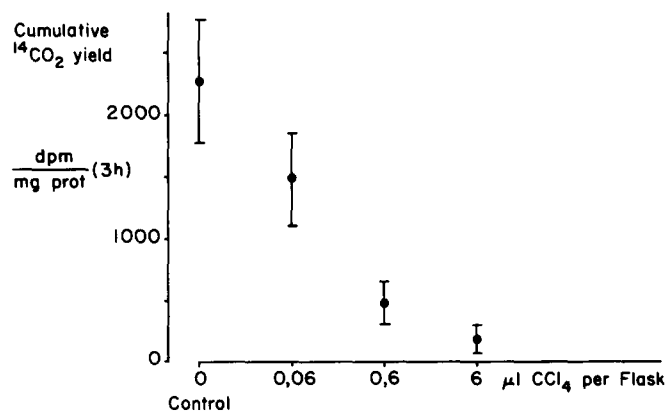

Fig. 3. Effect of CCl_4-induced cell damage on cumulative $^{14}CO_2$ yield collected during 3 hr after addition of tracer amounts of labelled aminopyrine. There is a dose-dependent depression of $^{14}CO_2$ production reflecting the toxic effect of CCl_4 on microsomal cell function. Each point represents the mean ± S.D. of 5 monolayers.

[^{14}C]aminopyrine was linear during 6 hr of collection. The effects of phenobarbital induction and testosterone inhibition, as well as dose-dependent CCl_4 toxicity, were clearly demonstrable and the system was suited for enzyme kinetic studies of aminopyrine *N*-demethylation. It is suggested that this simple and noninvasive approach may prove useful for the study of drug metabolism and drug toxicity in hepatocyte monolayers, and for the rapid assessment of culture viability.

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Department of Clinical Pharmacology
University of Berne
3010 Berne, Switzerland

STEPHAN HOTTINGER
WILHELM RÖLLINGHOFF
HUBERTUS WIETHOLTZ
RUDOLF PREISIG*

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* Author to whom all correspondence should be addressed.

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Cholate-salt solubilisation of bovine brain muscarinic receptors

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Muscarinic cholinergic receptors have been extensively characterised in their membrane state (for recent review see [1]). The presence of receptors can be detected *in vitro* using radioligands (usually antagonists such as propylbenzilycholine [2], quinuclidinyl benzilate [3], *N*-methyl-4-piperidylbenzilate [4]) which label a saturable high affinity binding component able to be specifically displaced by muscarinic agonists and antagonists. These antagonists bind to a single high affinity receptor site while strong agonists bind with a lower potency and give binding curves with Hill coefficients less than 1 suggesting a possible heterogeneity of binding sites of high and low affinities [5]. In order to understand the precise functioning of the muscarinic receptor it is necessary to study the receptor in an isolated state; however, purification attempts have been hampered by the lack of an efficient solubilising agent. Earlier attempts to solubilise the muscarinic receptor made use of high salt [6, 7] or the detergent digitonin [8]. More recently several other laboratories have characterised the receptors using this detergent; however, the binding characteristics of the soluble material varied between reports. In two reports, strong agonists no longer bound to multiple sites but instead to a single binding site of low affinity in one case [9] and of high affinity in the other [10]. The ability of digitonin to solubilise muscarinic receptors also appeared to vary between batches [11]. A more useful solubilising agent would combine a reproducible and efficient solubilisation of membrane-bound receptors with retention of muscarinic binding characteristics. Reported here is the solubilisation of specific [^{3}H]quinuclidinyl benzilate binding sites from bovine brain caudate nucleus using a combination of 0.1% sodium cholate and 1 M NaCl.

* Abbreviations: QNB, quinuclidinyl benzilate; EGTA, ethylene-glycol-bis (β-amino-ethyl ether) *N*,*N*′-tetraacetic acid.

Materials and methods

Ox brains were obtained fresh from the slaughterhouse within 4 hr of death. The caudate nucleus was dissected out and a microsomal pellet (1500–2500 pmoles [^{3}H]QNB* binding sites/g protein) was prepared as described in [12]. Protein was assayed as in [13].