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Altered responsiveness to alpha- and beta-adrenoceptor stimulation in hepatocytes cultured in defined medium

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Catecholamines regulate important metabolic pathways such as glycogenolysis and gluconeogenesis through alpha and beta adrenoceptors in the livers of a variety of species. In the adult rat liver, it is now well documented that the activation of glycogen phosphorylase by catecholamines is predominantly a cAMP-independent process mediated by alpha$_1$-adrenoceptor stimulation and is thought to be the result of an elevation in the concentration of intracellular Ca^{2+} [1–10]. However, beta-adrenoceptor-mediated activation of glycogen phosphorylase, mediated by cAMP-dependent processes, plays a relatively minor role in the adrenergic activation of glycogenolysis in normal adult rat liver. This pattern of adrenergic activation in adult rat liver with alpha much more prominent than beta is not fixed and static. Indeed, this pattern has been shown to change in a number of physiological and pathological conditions, including adrenalectomy [11], hypothyroidism [12], regeneration [13], cholestasis [14] and preneoplasia [15]. Also, it has been found that beta adrenergic activation of glycogen phosphorylase is more marked in livers from young rats [16].

Recently, it was reported that the relative activities of alpha and beta agonists are progressively reversed during the *in vitro* culturing of isolated hepatocytes [17, 18]. The primary role of alpha adrenoceptors in activating glycogen phosphorylase decreases and an effective beta response, absent in freshly isolated cells, emerges after primary culture. Our studies were designed to investigate the possibility that changes in alpha and beta receptors might underline these alterations in responsiveness.

Materials and methods

Materials. Unlabeled (±)-cyanopindolol was a gift from Dr. G. Engel of Sandoz (Basel, Switzerland). [^{125}I]Cyanopindolol ([^{125}I]CYP) was prepared essentially as described by Engel *et al.* [19]. Carrier-free Na^{125}I (Catalog No. IMS 30) was purchased from Amersham (Arlington Heights, IL). Alpha-D-[U-^{14}C]Glucose-1-phosphate and [^{3}H]prazosin were purchased from New England Nuclear (Boston, MA). Anti-cAMP* rabbit antiserum were obtained from Becton Dickinson. Prazosin was a gift from the Pfizer Pharmaceutical Co. Collagenase (Type II) was from the Worthington Biochemical Co. (Freehold, NJ); newborn calf serum, tissue culture medium 199 (Earl's salt), and DMEM were from Gibco. Other chemicals and reagents were purchased from standard commercial sources.

Isolation of hepatocytes. Male Sprague–Dawley rats, weighing 240–260 g, that had been fed *ad lib.* were used. Hepatocytes were isolated by the collagenase perfusion method of Berry and Friend [20] as modified by Bissell and Guzelian [21]. The yield of cells/liver averaged 1.5×10^8, with 90–95% viability as estimated by trypan blue exclusion.

Fresh hepatocytes. Freshly isolated hepatocytes (6 to 15×10^6 cells) were incubated in plastic tubes in a total volume of 1 ml of Hepes-buffered medium (134 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) supplemented with 40 mM glucose and shaken in an orbital water bath shaker at 150–200 rpm for 30 min at 37°. At the end of the incubation, 200-μl aliquots of the cell suspension were transferred to plastic tubes containing 50 μl of drugs as indicated. The tubes were then shaken in a bath at 37° for 2 min [19] and immediately immersed in liquid nitrogen to terminate the reaction. The frozen hepatocytes were stored at −80° until assay. For the glycogen phosphorylase assay, the frozen hepatocytes were first mixed with a half volume of 30 mM MOPS, 150 mM NaF, 15 mM EDTA and 3 mM dithiothreitol (pH 7.0). The samples were homogenized with a Polytron cell disrupter (Brinkmann Instruments, Westbury, NY) at setting 8 for 20 sec. The homogenates were centrifuged at 12,000 *g* for 5 min at 4°, and the supernatant fraction was used for the assay of glycogen phosphorylase and cAMP as described below.

Cultured hepatocytes. For cultured hepatocytes, Medium 199 was supplemented with the following: 100 units/ml penicillin G; 100 μg/ml streptomycin sulfate: 26 mM $NaHCO_3$; an additional 10 mM glucose (final concentration, 15.6 mM); 3×10^{-8} M crystalline insulin; and 1×10^{-6} M corticosterone. Approximately 4×10^6 cells in a final volume of 3.0 ml were placed in a 60 mm culture dish coated with collagen that had been purified and solubilized by the method of Wood and Keech [22]. The cultures were maintained at 37° in an atmosphere of 95% air and 5% CO_2. The cultured medium was changed to the Hepes-buffered medium described above supplemented with 40 mM glucose at the end of 24 hr. Culture dishes were then incubated at 37° in an atmosphere of 95% air and 5% CO_2 for 30 min. After this preincubation, 50-μl aliquots of various drugs were added to the culture dishes and incubated for 2 min at 37°. At that point, the medium was immediately discarded and the dishes were placed on the dry ice to terminate the reaction. The frozen hepatocytes attached to the dish were stored at −80° until assay.

* Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; [^{125}I]CYP, [^{125}I]cyanopindolol; MEM, Minimum Essential Medium; A23157, calcium ionophore, A23187; IBMX, 3-isobutyl-1-methylxanthine; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and DMEM, Dulbecco's Minimum Essential Medium.

To prepare the samples for assay, the frozen cultured hepatocytes were thawed on ice and 5 ml of ice-cold buffer (0.5 mM MOPS, 2.5 mM NaF, 0.25 mM EDTA, 0.05 mM dithiothreitol, pH 7.0) was added to the dish. The cells were scraped from the dish and homogenized with a Polytron cell disruptor at setting 8 for 20 sec. The homogenates were centrifuged at 12,000 *g* for 5 min at 4°. The supernatant fraction was then frozen in liquid nitrogen and lyophilized. The resulting sample was resuspended in 250 μl of ice-cold 60 mM MES buffer (pH 6.1). Lyophilization of the samples did not affect either cAMP levels or glycogen phosphorylase activity.

Assays of glycogenolysis and cAMP formation. Glycogen phosphorylase *a* activity was measured using a filter disc assay similar to that of Gilboe *et al.* [23] as modified [24]. Cyclic AMP was measured by radioimmunoassay [25].

Hepatocyte membrane preparation for radioligand binding assays. Freshly isolated and cultured cells (about 7×10^7 cells each) were suspended in 20 ml of ice-cold buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.5) and homogenized with a Polytron at setting 8 for 20 sec. The homogenates were centrifuged at 20,000 *g* for 30 min. The pellet was resuspended in 50 mM Tris–HCl (pH 7.4) containing 10 mM $MgCl_2$ and used immediately in the receptor binding assays.

$Alpha_1$-receptor number was determined by incubating 100 μl of the membrane suspension in a total volume of 150 μl containing [^{3}H]prazosin (0.1 to 4 nM). Incubations were continued for 30 min at 37° by which time equilibrium had been achieved. Nonspecific binding was defined as binding remaining in the presence of 10 μM phentolamine. The specific binding of [^{3}H]prazosin was shown to have appropriate specificity [26].

Beta-adrenergic receptor assays using [^{125}I]CYP (40–600 pM) contained a total volume of 150 μl; the incubation was for 60 min at 25° by which time binding was at equilibrium. The specific binding was defined as the difference in the amount of bound ICYP obtained in the absence and in the presence of 1 μM (±)-propranolol. Specific ICYP binding sites had the expected beta-adrenergic specificity and stereospecificity.

Protein concentration was determined by the method of Lowry *et al.* [27] using bovine serum albumin as standard.

Data analysis. The data from saturation curves of radioligand studies were analyzed using nonlinear regression on a HP9816 computer. These data were fit based on the law of mass action using a general program for the analysis of data in terms of models. The experimental data given in the text are expressed as the mean ± S.E.M.

Results

Our initial experiments were designed to compare activation of glycogen phosphorylase in freshly isolated hepatocytes versus hepatocytes that had been cultured for 24 hr. As indicated in Fig. 1, in freshly isolated hepatocytes the alpha-selective adrenergic agonist phenylephrine activated glycogen phsophorylase effectively. Furthermore, the $alpha_1$ selective antagonist prazosin inhibited the activation of glycogen phosphorylase by phenylephrine, epinephrine and a high concentration of isoproterenol (10^{-4} M). The beta-antagonist propranolol was relatively ineffective in blocking the effect of these agonists. Therefore, these data suggest that $alpha_1$-receptor stimulation of glycogen phosphorylase predominates over beta receptor activation in freshly isolated hepatocytes as has been seen previously [2, 5, 28]. In hepatocytes that had been cultured for 24 hr the pattern of responsiveness changed markedly. In the cultured cells, isoproterenol effectively activated glycogen phosphorylase (Fig. 1). The stimulation by isoproterenol was blocked by propranolol but not prazosin. The activation by phenylephrine and epinephrine was partially blocked by both propranolol and prazosin, suggesting that these agonists were activating glycogen phsophorylase through both alpha and beta receptors in the cultured cells. There was greater stimulation above basal by phenylephrine (10^{-5} M) in the freshly isolated hepatocytes than in the cultured cells (5.9 ± 1.0 vs 1.8 ± 0.4 units/100 mg protein respectively), indicating a loss in alpha stimulation. Conversely, beta-receptor-mediated stimulation by isoproterenol (10^{-5} M) increased from 0.3 ± 0.1 units/100 mg protein in the fresh cells to 3.9 ± 1.1 units/100 mg protein in the cultured hepatocytes.

We examined the activation of glycogen phosphorylase by vasopressin [29] and the ionophore A23157 [30] which both produce increases in intracellular Ca^{2+} in fresh and cultured hepatocytes. The ability of vasopressin to activate glycogen phosphorylase actually increased in the cultured cells, and there was no change in the maximal responsiveness to A23157 (data not shown). These data indicated that there was no evidence for a general decline in cAMP-independent activation of glycogen phsophorylase and suggested some specificity in the loss in responsiveness to alpha-adrenergic stimulation. Consequently, we measured alpha receptors in membranes prepared from fresh and cultured hepatocytes with [^{3}H]prazosin. There was a marked decrease in the number of alpha-adrenergic receptors in the membranes prepared from control hepatocytes (Fig. 2). These data suggest but do not definitely establish

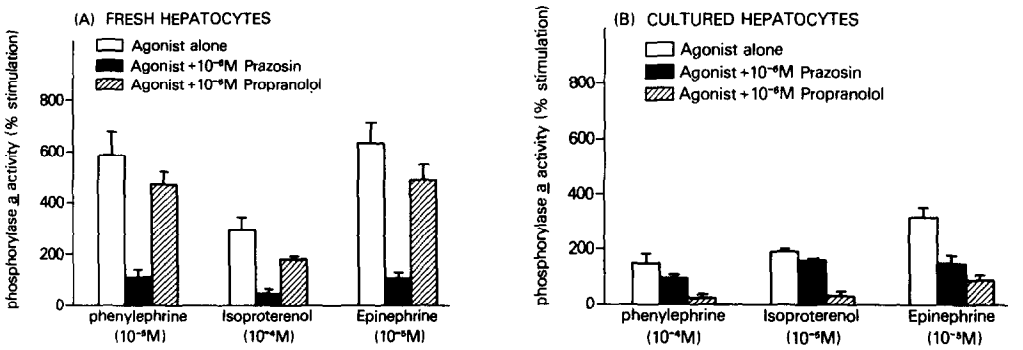

Fig. 1. Stimulation of glycogen phosphorylase in freshly isolated (A) and cultured (B) hepatocytes from adult male rats by phenylephrine, isoproterenol and epinephrine, and the influence of alpha- and beta-adrenoceptor antagonists. Hepatocytes were preincubated for 15 min with 1 μM prazosin or 1 μM propranolol. Then agonists were added as indicated. The results are presented as the mean ± S.E.M. of results obtained from at least three separate experiments.

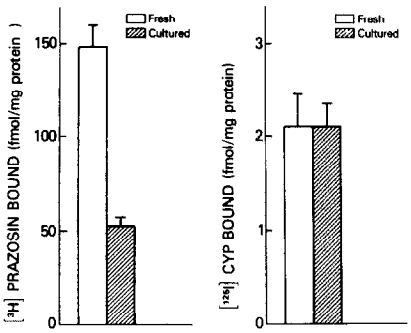

Fig. 2. Specific binding sites of [^{3}H]prazosin and [^{125}I]-CYP in membranes from freshly isolated and cultured rat hepatocytes. Data for figure are the mean ± S.E.M. of four separate experiments.

that the loss in responsiveness to alpha$_1$ agonists was caused by a loss in alpha$_1$ receptors.

With culture, there was a marked development in the ability of beta-adrenergic agonists to stimulate glycogen phosphorylase (Fig. 1). Also, isoproterenol did not activate cAMP accumulation in the fresh cells, whereas it caused a marked stimulation in the cultured hepatocytes (data not shown). In an effort to determine the mechanism for the development of the beta-adrenergic response, we measured beta-adrenergic receptors in membranes from fresh and cultured hepatocytes with the antagonist [^{125}I]CYP. We found no change in either the number of beta receptors (2.1 ± 0.4 vs 2.1 ± 0.3 fmoles/mg protein) or in their affinity for [^{125}I]CYP (K_D 114 ± 8 vs 118 ± 15 pM) in the membranes from fresh and cultured hepatocytes respectively (Fig. 2). Consequently, the development of beta-adrenergic stimulation of cAMP accumulation and glycogen phosphorylase activation cannot be explained by a simple change in beta-adrenergic receptor number.

Nakamura *et al.* [31] and Refsnes *et al.* [32] have previously reported development of enhanced adrenoceptor beta responsiveness in cultured hepatocytes. Both those groups found an increase in the number of beta receptors in membranes from cultured hepatocytes [31, 32]. In an effort to reconcile our results with their findings, we conducted further experiments to explore some obvious methodological differences. First, Nakamura *et al.* [31] utilized a highly purified membrane preparation. We replicated that membrane preparation and found a similar enrichment in the density of beta-adrenergic receptors, 20.9 fmoles/mg protein (N = 2), in membranes from fresh hepatocytes as reported by Nakamura *et al.* However, the density of beta receptors in enriched membranes from cultured hepatocytes, 17.8 fmoles/mg protein (N = 2), was similar to the result in the fresh hepatocytes. Consequently, the different membrane preparation did not appear to explain the discrepancy between our results and those of Nakamura *et al.* Also, Refsnes *et al.* [32] used a crude membrane preparation similar to that used in our studies. Another major experimental difference is that our cultures were conducted in defined medium whereas those studies [31, 32] were done in the presence of serum. Therefore, we undertook further experiments using freshly isolated hepatocytes and cultured hepatocytes grown in DMEM with 10% newborn calf serum and dexamethasone (10^{-6} M) using conditions similar to those in Ref. 31. In a mean of four experiments, the beta receptors increased from 2.1 ± 0.4 fmoles/mg protein in membranes from hepatocytes grown in defined medium to 5.3 ± 0.8 fmoles/mg protein in membranes from hepatocytes cultured in medium containing serum ($P < 0.05$). There was no change in the K_D of [^{125}I]CYP (116 ± 12.0 vs 112 ± 11.8 pM) in the two groups. Also, there was no difference in the development of the enhanced ability of isoproterenol to stimulate cAMP accumulation whether the cells were grown in serum or defined medium (Table 1).

Discussion

In the livers from neonatal rats, beta receptors play a prominent role in mediating catecholamine effects [33, 34]. However, in the adult rat liver, beta responsiveness is minimal and glycogen phosphorylase is activated by alpha$_1$ receptors [35]. Ichihara *et al.* [17] made the surprising observation that, after primary culture, hepatocytes became more responsive to beta-adrenoceptor agonists. Okajima and Ui [18] showed that, after a short period in primary culture, not only was there an increase in beta-receptor stimulation but also a decline in alpha-receptor efficacy. We have largely confirmed those observations regarding the change in pattern of catecholamine activation of glycogen phosphorylase in cultured hepatocytes.

There was a decline in the ability of alpha$_1$ receptors to activate glycogen phosphorylase in hepatocytes cultured for 24 hr, whereas responses to vasopressin and A23157 were preserved, indicating that there was some specificity to the loss in responsiveness to alpha$_1$ stimulation. However, one cannot conclude that the loss in alpha$_1$ receptors was the actual cause of the blunted response since other factors may also have been involved, such as coupling of the alpha$_1$ receptors to their effector.

The explanation for the acquisition of the beta-adrenoceptor response after culture is much less clear. Our data and those of others [17, 18, 31, 32, 36–38] indicate that in adult hepatocytes beta agonists cause only a small increase in cAMP accumulation and glycogen phosphorylase activation, whereas after primary culture the cAMP and glycogen phosphorylase responses are greatly increased. Presumably, it is the augmented cAMP response which enables beta agonists to stimulate glycogen phosphorylase. The

Table 1. cAMP accumulation in cultured hepatocytes grown in the presence or absence of serum

	cAMP accumulation (pmoles/mg protein/2 min)	
	Serum-free medium	Serum-containing medium
Basal	14.1 ± 0.6	10.6 ± 1.5
Isoproterenol (10^{-5} M)	61.7 ± 4.6	60.3 ± 4.1
Glucagon (10^{-7} M)	57.5 ± 4.6	57.6 ± 12.7

Isolated hepatocytes were grown in serum-free Medium 199 as described in Materials and Methods or in DMEM containing 10% newborn calf serum as described in Results. Stimulation of cAMP accumulation was done in cells washed after 24 hr in culture in the presence of 0.2 mM IBMX. Values are means ± S.E.M., N = 4.

mechanism for the enhanced cAMP response is uncertain. Nakamura *et al.* [31] found no change in the amount of stimulatory guanine nucleotide regulatory protein (N_s) labeled by cholera toxin in cultured hepatocytes. An obvious hypothesis to explain the augmented beta response is that there is an increase in beta adrenoceptors in the cultured hepatocytes. Indeed, three groups have found an increase in beta adrenoceptors in cultured hepatocytes [31, 32, 36, 38], although Kunos *et al.* [37] did not. In our initial experiments we found no change in the number of beta adrenoceptors. We have examined certain methodological differences between our work and studies recently reported by others [31, 32]. Utilizing the membrane purification scheme of Nakamura *et al.* [31], we were able to reproduce the beta-receptor density found by those workers in control hepatocyte membranes, but we still found not increase in this number after primary culture when we made this technical alteration. A more likely explanation for the differences in results is the use of serum rather than defined medium in the hepatocyte culture. These other groups primarily utilized serum in their cultures, whereas we utilized defined medium in our initial experiments. When we conducted cultures in 10% newborn calf serum, we found an augmentation in cAMP response to beta agonists similar to that we had seen in cells grown in defined medium (Table 1). However, the cells grown in serum-containing medium had a significant increase in beta-receptor number. These data suggest that an increase in beta receptors is not necessary for the development of the enhanced cAMP response. Our results suggest that an increase in beta-receptor number is a separate process due to factor(s) present in serum. However, Schwarz *et al.* [38] compared serum to defined medium and found similar increases in beta adrenoceptors under the two conditions. We cannot readily explain the difference in our results compared with theirs. The possibility that serum may modify beta adrenoceptors has been documented previously in transformed cells in culture [39, 40].

We have fortuitously found experimental conditions where the augmented cAMP response in cultured hepatocytes can be dissociated from an increase in beta-adrenoceptor number. Consequently, our results suggest that an alteration in some other property of the hepatocytes is responsible for the enhanced response to isoproterenol. One could conjecture that a change in the stimulatory regulation of adenylate or conceivably a release of inhibitory effects on adenylate cyclase occurs. For example, Garcia-Sainz and co-workers [14] demonstrated that pertussis toxin enhances beta-adrenoceptor stimulation in hepatocytes, suggesting that inactivation of inhibitory guanine nucleotide regulatory protein (N_i) has an important effect in this system. Indeed, Itoh *et al.* [42] have reported that there is a parallel loss over time in a component of N_i (labeled by ADP-ribosylation in the presence of pertussis toxin) and the development of beta-adrenergic receptor-stimulated cAMP accumulation in cultured hepatocytes. Furthermore, Kunos *et al.* [37] have shown recently that, after 4 hr in culture in serum-free medium, there is a development of enhanced beta-adrenoceptor responsiveness in hepatocytes with no change in receptor number. Those workers conjecture that the increased efficacy of the beta receptors is due to changes in membrane phospholipase A_2 activity.

Our results further characterize the extensive alterations in responses to catecholamines that occur in primary culture of hepatocytes. The results illustrate the caution that must be used in interpretation of changes in receptor number when trying to explain altered physiological responses. Enhanced efficacy of beta-adrenoceptor-mediated activation of glycogen phosphorylase can occur in the absence of an increase in receptor number.

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Studies on rat liver cytochrome P-450s involved in the metabolism of antipyrine: phenobarbital- and 3-methylcholanthrene-inducible isozymes possessing 4-hydroxylase activity

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Antipyrine (AP)* plasma half-life or clearance has been used widely to assess the drug metabolizing activity in man [1, 2], although the ability to metabolize AP is not always correlated with that of other drugs [3, 4]. AP is metabolized to 4-hydroxy [5], *N*-desmethyl [6], 3-hydroxymethyl [7], 3-carboxy [8], 3,4-dihydrodihydroxy [9] and 4,4′-dihydroxy [10] derivatives in mammals (Fig. 1).

Several workers have already suggested that these metabolic pathways are catalyzed by different forms of cytochrome P-450 [11–16]. This, however, was introduced mainly by *in vivo* studies showing different magnitudes of enhancement of the above metabolic reactions by pretreatment with typical inducers such as phenobarbital (PB) and 3-methylcholanthrene (MC). In the present study, the role of cytochrome P-450s induced by pretreatment with PB and MC in the 4-hydroxylation and 3-methyl hydroxylation of AP was investigated using liver microsomes and purified cytochrome P-450 isozymes from inducer-pretreated rats.

Materials and methods

Chemicals. AP was obtained from a commercial source. 4-Hydroxy-AP and 3-hydroxymethyl-3-nor-AP were isolated from urine of rats treated with AP in our laboratory [7]. All other reagents used were from the sources described elsewhere [17, 18] or of the highest quality commercially available.

Purification of enzymes. Major forms of cytochrome P-450, which are induced with PB and MC, were partially purified from livers of male Sprague–Dawley rats (150–250 g) by the method of Guengerich and Martin [19] with some modifications. The fraction designated as the B_2 fraction by the above workers was further purified as follows. In the case of PB-pretreated animals, this fraction was found to contain two distinct forms of cytochrome P-450 (P-450-SD-I and -II). After dialysis of the B_2 fraction against 20 mM potassium phosphate (pH 7.25) containing 20% (v/v) glycerol, the above isozymes were separated by a carboxymethyl Sephadex C-50 (Pharmacia Fine Chemicals) column chromatography using the same buffer as a effluent. While almost all of the P-450-SD-I was adsorbed, P-450-SD-II passed through the column. Purified P-450-SD-I was then obtained by eluting the column with 150 mM potassium phosphate (pH 7.25) containing 20% (v/v) glycerol. On the other hand, P-450-SD-II was dialyzed against 5 mM potassium phosphate containing 20% (v/v) glycerol, and purified by rechromatography with carboxymethyl Sephadex C-50 column using the dialyzing buffer as an effluent. Purification of MC-inducible cytochrome P-450 (P-450-SD-III) from the B_2 fraction was carried out by the method described previously [20] using hydroxylapatite column. The specific contents of P-450-SD-I, -II and -III were shown to be 14.1, 10.0 and 15.1 nmole P-450/mg protein, respectively.

NADPH-cytochrome P-450 reductase (34.3 μmole cytochrome *c* reduced/min/mg protein) was isolated from PB-treated rats by the method of Guengerich and Martin [19] with some modifications. One unit of the reductase was defined as activity of 1 μmole cytochrome *c* reduced/min/mg protein.

Determination of hydroxylase activity for antipyrine. Assay of AP 4-hydroxylase and 3-methyl hydroxylase in liver microsomes was conducted in the incubation mixture consisting of 20 μmole of AP, microsomes containing about 4 mg protein, 0.66 μmole of NADP, 16 μmole of glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 12 μmole of magnesium chloride and 50 mM of Tris–HCl (pH 7.4) to make a final volume of 2 ml. After incubation for 12.5 min at 37°, the reaction was terminated by ice-cooling, and *p*-phenetidine (1.6 μg), an internal standard, was added to the solution. The incubation mix-

* Abbreviations used: AP, antipyrine; PB, phenobarbital; MC, 3-methylcholanthrene.