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ACCUMULATION OF PHENOLS AND CATECHOLS IN ISOLATED MOUSE HEPATOCYTES IN STARVATION OR AFTER PRETREATMENT WITH ACETONE

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Abstract—Conditions leading to the accumulation of unconjugated phenols and catechols were investigated in mouse livers. The formation of unconjugated hydroxylated products of added *p*-nitrophenol and aniline was investigated in isolated hepatocytes prepared from 48 hr fasted or fed mice or from fed mice after acetone pretreatment. 4-Nitrocatechol and *p*-aminophenol — the hydroxylated products of *p*-nitrophenol and aniline — were accumulated in cells prepared from fasting animals, while in cells prepared from fed mice these unconjugated derivatives were not detectable. The accumulation of 4-nitrocatechol and *p*-aminophenol was also shown in isolated hepatocytes prepared from acetone pretreated fed mice. Inhibition of glucuronidation by N^6,O^2-dibutyryl cAMP or by D-galactosamine increased the accumulation of 4-nitrocatechol upon addition of *p*-nitrophenol in cells prepared from fasted mice. Both 48 hr starvation and acetone pretreatment enhanced the activity of microsomal *p*-nitrophenol and aniline hydroxylase by 300% and 600%, respectively, whereas *p*-nitrophenol conjugation in isolated hepatocytes as well as in hepatocyte homogenates was decreased by about 80% after 48 hr starvation. Acetone pretreatment did not alter the rate of *p*-nitrophenol conjugation measured in liver homogenates. It is suggested that a shift from conjugation toward hydroxylation in starvation gives rise to the formation of hazardous metabolites.

Formation of catechols from aromatic compounds in liver parenchymal cells can lead to liver injuries [1]. Accumulation of phenols and catechols is a dangerous condition; catechols are readily oxidized to semiquinones and quinones, which are regarded to be toxic metabolites [2–4]. Phenols are conjugated rapidly and to a large extent [5] and therefore, they are not accumulated and not hydroxylated to form catechols in liver cells under normal conditions. Nevertheless, it has been reported that in liver microsomes 4-nitrocatechol can be formed from *p*-nitrophenol [6]. Hydroxylation of *p*-nitrophenol is catalyzed by a cytochrome P-450 isoenzyme belonging to P-450 II E gene subfamily [7] designated form 3a in rabbits and P-450j in rats [8–10]. Cytochrome P-450j has been shown to catalyze also the hydroxylation of different other phenols, e.g. aniline and the oxidation of acetone and ethanol. Microsomal hydroxylation of *p*-nitrophenol by cytochrome P-450j is highly inducible by ethanol [11, 12]. This enzyme is also induced by fasting or by addition of isoniazid in rats and rabbits; its activity is elevated in diabetes or after acetone treatment [8–14]. In mice an increase of aniline hydroxylase activity has been demonstrated also in diabetes or after starvation and upon glucagon infusion [15] suggesting the existence of a murine cytochrome P-450j like isozyme.

Nutritional parameters have been shown to alter drug metabolism and also drug toxicity. Fasting can alter the activity of several enzymes involved in biotransformation depending on species, sex, type of drugs etc., as well as the supply of cofactors (NADPH, UDP-glucuronic acid etc) required for various phases of biotransformation [16, 17]. Biotransformation is an oxygen and energy consuming process in the liver. Thus, it is in a close interrelationship with several other pathways of the hepatic intermediary metabolism (gluconeogenesis, ureogenesis, lipid synthesis [16–21]), which are — at least partly — under a cAMP-dependent control. Recently it has been shown that during starvation the priority of glucose production is guaranteed not only by a cAMP-dependent increase of gluconeogenesis but also by a cAMP-dependent negative regulation of certain processes of drug oxidation (aminopyrine *N*-demethylation) and conjugation (*p*-nitrophenol glucuronidation) [21]. However, a negative cAMP-dependent regulation could not be evidenced in the case of aniline hydroxylation. Since the enzyme responsible for *p*-nitrophenol and aniline hydroxylation could be induced by fasting and acetone pretreatment, it seemed interesting to examine whether these conditions led to an increased production of catechols and phenols from aniline or *p*-nitrophenol. It has been shown that *p*-nitrophenol itself or its hydroxylated product undergoes further conjugation [6, 11]. Conjugation of *p*-nitrophenol could be inhibited by cAMP-dependent phosphorylation [21], therefore, in starvation there was also a possibility that in addition to the enhanced hydroxylation conjugation might be decreased favoring the accumulation of the hydroxylated products.

It has been found that in starvation the potentially hazardous 4-nitrocatechol and *p*-aminophenol accumulate in isolated mouse hepatocytes upon addition of *p*-nitrophenol or aniline. The accumu-

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lation is mainly the consequence of the increased hydroxylase activities; however, a contribution of a decreased conjugation to the increase of 4-nitrocatechol formation has also been detected. The accumulation of unconjugated phenols or catechols upon addition of *p*-nitrophenol and aniline can also be demonstrated in isolated hepatocytes prepared from fed mice; however, only after acetone pretreatment.

MATERIALS AND METHODS

Isolated hepatocytes were prepared from male CFLP mice fed *ad lib.* or starved for 48 hr with the collagenase perfusion method as detailed previously [22]. In a series of experiments isolated hepatocytes were prepared from mice pretreated with acetone (added to drinking water (1%; v/v) with free access to standard food) for 5 days [23]. Hepatocytes (5×10^6 cells/ml) were incubated in Krebs–Henseleit bicarbonate buffer pH 7.4 containing 1% albumin, 8.5 mM glucose, 5 mM pyruvate and amino acids necessary for protein synthesis (1 mM of each) under constant bubbling of gas (O_2:CO_2, 95:5, v/v) at 37°. In cases when the cells were prepared from fasted animals glucose, pyruvate and amino acids were omitted from the incubation medium.

Oxidation of aniline and *p*-nitrophenol in isolated hepatocytes was determined by determination of *p*-aminophenol [24] or 4-nitrocatechol [25]. Conjugation of *p*-nitrophenol and phenolphthalein in isolated hepatocytes was measured as described earlier [21].

Washed hepatic microsomes were prepared by differential centrifugation. Aniline hydroxylase and *p*-nitrophenol hydroxylase activities in liver microsomes were assayed by [26].

p-Nitrophenol UDP glucuronosyltransferase activity was measured in homogenate of hepatocytes as described by Bock *et al.* [27].

Protein contents were measured by the method of Lowry *et al.* [28]. DNA contents were determined by the method of Burton [29].

RESULTS

Effect of starvation on p-nitrophenol and aniline hydroxylation in isolated mouse hepatocytes

Isolated hepatocytes prepared from fed or fasted mice were incubated in the presence of 0.1 mM *p*-nitrophenol or 2 mM aniline and the formation of the hydroxylated products, 4-nitrocatechol or *p*-aminophenol was measured. Figures 1A and 1B show that 4-nitrocatechol or *p*-aminophenol were not detectable under our experimental conditions in suspension of isolated hepatocytes prepared from fed mice in a 60-min incubation. If the cells were prepared from 48 hr fasted mice the formation of 4-nitrocatechol or *p*-aminophenol was linear in time.

An accumulation of unconjugated hydroxylated products might be a consequence of an increased hydroxylation and/or a decreased conjugation. To investigate these possibilities first accumulation of the unconjugated hydroxylated *p*-nitrophenol

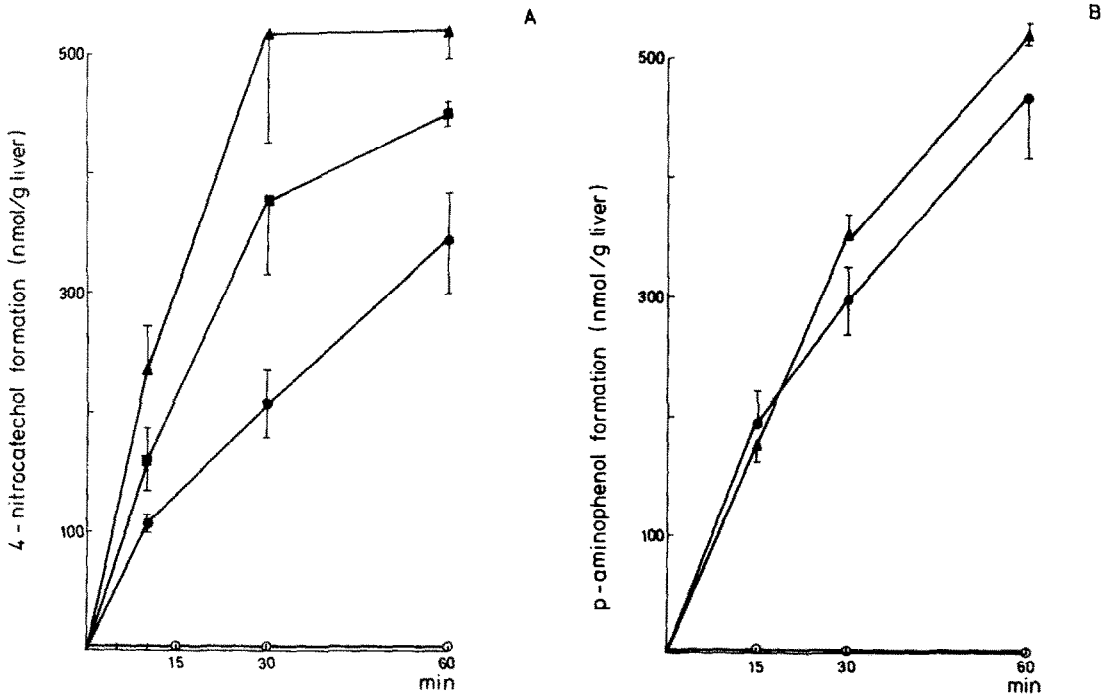

Fig. 1. 4-nitrocatechol and *p*-aminophenol formation in isolated hepatocytes. Isolated hepatocytes were prepared from 48 hr starved mice. The cells were incubated with 100 μM *p*-nitrophenol (A) or 2 mM aniline (B) and the formation of 4-nitrocatechol (A) or *p*-aminophenol (B) was measured in the presence of 1 mM dibutyryl cAMP (▲), 10 mM D-galactosamine (■) or without additions (●). 4-Nitrocatechol (A) or *p*-aminophenol (B) formation of isolated hepatocytes prepared from fed mice (○) was also determined. Mean ± SD (N = 4).

Table 1. Effect of starvation on conjugation in isolated mouse hepatocytes

	p-Nitrophenol	Phenolphthalein
	Disappearance (nmol/min/g liver)	
Fed	44.7 ± 3.9	30.9 ± 8.2
Fasted	9.5 ± 2.0	3.8 ± 0.3

Isolated hepatocytes were prepared from mice starved for 48 hr or fed *ad lib*. The cells were incubated with 100 μM *p*-nitrophenol or 100 μM phenolphthalein and the disappearance of the aglycones was determined. Mean ± SD (N = 8).

metabolite 4-nitrocatechol was measured in the presence of D-galactosamine known to cause serious uridylate deficiency and as a consequence inhibition of glucuronidation in isolated hepatocytes [30–32, 21]. 4-Nitrocatechol accumulation was markedly increased by 10 mM galactosamine in hepatocytes from fasted mice (Fig. 1A). It is noted that only a negligible galactosamine effect was shown in hepatocytes prepared from fed mice (data not shown). It was also of interest to examine the effect of dibutyryl cAMP, which has been also shown to inhibit *p*-nitrophenol glucuronidation [21]. 4-Nitrocatechol accumulation was also increased by 1 mM dibutyryl cAMP in hepatocytes prepared from starved mice (Fig. 1A). Dibutyryl-cAMP caused only a minor increase in the accumulation of *p*-aminophenol in cells prepared from fasted mice (Fig. 1B).

Nevertheless, the question still remained open, whether these increased hydroxylations were accompanied by changes in glucuronidation as well.

Effect of starvation on p-*nitrophenol conjugation*

Isolated hepatocytes prepared from fed or starved mice were incubated in the presence of 100 μm *p*-nitrophenol or 100 μM phenolphthalein. At these substrate concentrations mainly glucuronides are formed as conjugates [33] but the glucuronidations of *p*-nitrophenol and phenolphthalein are catalyzed by different UDP-glucuronosyl transferases [27]. Starvation caused an 80% or 90% inhibition of *p*-nitrophenol or phenolphthalein conjugation, respectively (Table 1). Therefore, it was suggested that the shortage of UDP-glucuronic acid caused by starvation *per se* might be responsible for the decreased conjugation.

To rule out this possibility the activity of *p*-nitrophenol UDP-glucuronosyl transferase was measured in liver homogenates of fed or 48 hr fasted mice in the presence of UDP-glucuronic acid. Starvation caused a 75% decrease of *p*-nitrophenol glucuronidation compared to the fed state (Table 2).

This decreased activity of *p*-nitrophenol glucuronosyl transferase was accompanied by an enhanced *p*-nitrophenol hydroxylase activity. Microsomal membranes prepared from livers of fed, fasted or acetone pretreated fed mice were incubated in the presence of *p*-nitrophenol and the formation of 4-nitrocatechol was detected. Starvation caused an almost threefold increase in the formation of 4-nitrocatechol (Table 2). Similar changes were shown in the case of aniline. Acetone pretreatment resulted in a further increase of aniline and *p*-nitrophenol hydroxylase activities compared to starvation (Table 2). However, the activity of *p*-nitrophenol glucuronosyl transferase was not altered after pretreatment with acetone. It was of interest to examine whether the acetone pretreatment caused an accumulation of unconjugated catechols or phenols in hepatocytes from fed animals.

Effect of acetone pretreatment on aniline and p-*nitrophenol hydroxylation in isolated hepatocytes*

Isolated hepatocytes were prepared from fed mice pretreated with acetone or without any pretreatment. The cells were incubated in the presence of 0.1 mM *p*-nitrophenol or 2 mM aniline. Figure 2A shows that acetone pretreatment caused an enhanced formation of 4-nitrocatechol and the obtained values were higher compared to those in cells from starved animals (Fig. 1). Similar changes were seen in case of aniline hydroxylation (Fig. 2B). It is noted that when the cells were prepared from starved acetone pretreated mice a further increase of *p*-aminophenol accumulation occured compared to the fed acetone pretreated mice (data not shown).

Table 2. Effect of starvation and acetone pretreatment on aniline-, *p*-nitrophenol hydroxylase and *p*-nitrophenol UDP-glucuronosyl transferase activity in the liver

	Aniline hydroxylase	*p*-Nitrophenol hydroxylase	*p*-Nitrophenol glucuronosyl transferase
	(nmol/min/mg protein)		
Fed	1.124 ± 0.203 (5)	1.143 ± 0.186 (5)	3.53 ± 0.41 (6)
Fasted	3.395 ± 0.527 (5)	3.046 ± 0.265 (5)	0.93 ± 0.38 (4)
Fed, pretreated with acetone	6.020 ± 0.251 (6)	6.790 ± 0.506 (6)	3.53 ± 0.65 (6)

Microsomes were prepared from livers of fasted (48 hr), fed or acetone treated (5 days) fed mice. Activity of *p*-nitrophenol and aniline hydroxylase were determined. In a series of experiments the cells were homogenized and the activity of *p*-nitrophenol glucuronosyl transferase was determined in the presence of exogenous UDP-glucuronic acid. Values are expressed as nanomoles respective product formed/min/mg protein ± SD.

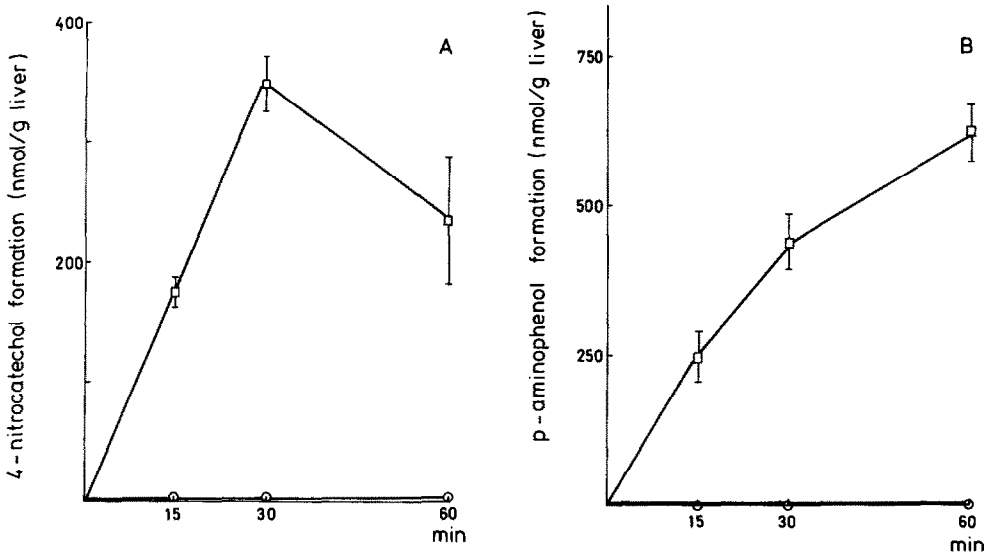

Fig. 2. Effect of acetone pretreatment on 4-nitrocatechol and 4-aminophenol formation in isolated hepatocytes. Isolated hepatocytes were prepared from fed mice pretreated with acetone for 5 days (□) or without any pretreatment (○). The cells were incubated with 100 μM *p*-nitrophenol (A) or with 2 mM aniline (B) and the formation of 4-nitrocatechol (A) or 4-aminophenol (B) was determined. Mean ± SD (N = 4).

DISCUSSION

In this study on the metabolism of *p*-aminophenol and aniline (widely used substrates of cytochrome P-450 II E subfamily enzymes), it is demonstrated that starvation affects the whole pattern of biotransformation. In fed animals the capacity of conjugation surpasses the capacity of oxidation; therefore, in isolated hepatocytes no accumulation of hydroxylated products of aniline or *p*-nitrophenol can be determined (Fig. 1). During starvation the balance is shifted towards the phase I oxidations and this way potentially toxic intermediates are accumulated. Similar changes can be elicited also in fed animals by acetone pretreatment (Fig. 2, Table 2). Phenols can be hydroxylated to catechols (or hydroquinones), aromatic amines to aminophenols. Both products can be autooxidized giving rise to quinones or quinone imines. These derivatives entering a redox cycle can cause cell injury. Semiquinone radicals can bind covalently to proteins and nucleic acids. Oxygen radicals formed can lead to peroxidation of microsomal lipids, to inactivation of enzymes and to breaks in DNA strands [3, 4]. Thus, the altered drug metabolism caused by starvation and also by acetone pretreatment (Fig. 2) is of toxicological importance. The possible toxicological implications of increased activity of cytochrome P-450 II E subfamily enzymes — responsible for *p*-aminophenol and aniline hydroxylation — even in humans have been already shown in chemical carcinogenesis by *N*-nitrosodimethylamine [13, 34, 35]. It has been also reported that chronic ethanol treatment (14 days) can increase aniline hydroxylase [36] and *p*-nitrophenol hydroxylase activities [11, 12]; therefore, it can be supposed that ethanol pretreatment may also result in an accumulation of various hydroxylated products. However, the accumulation of hydroxylated products of aniline and *p*-nitrophenol caused by starvation occurs already within 48-hr.

Enzymes engaged in biotransformation are regulated at transcriptional and post-translational levels [7, 14, 16]. The consequences of starvation on biotransformation seem to be manifested in changes of enzyme induction. Experiments presented in this paper confirm previous observations according to which *p*-nitrophenol and aniline hydroxylase are induced in starvation (Table 2). On the other hand, the role of cAMP in the molecular mechanisms of post-translational regulations in starvation has been widely studied. In our previous paper a negative cAMP-dependent control of certain processes of drug oxidation and glucuronidation has been reported in a close relationship with the well known positive cAMP dependent regulation of gluconeogenesis [21]. However, both aniline and *p*-nitrophenol hydroxylase activities have been found not to be under a negative cAMP-dependent control [21]. Alternatively, glucuronidation of *p*-nitrophenol has been decreased by a cAMP-dependent phosphorylation. Thus, in case of *p*-nitrophenol addition there is an increased oxidation controlled at the transcriptional level but the cAMP-dependent negative post-translational regulation in phase I of biotransformation is lacking. At the same time the cAMP dependent negative post-translational regulation in the II conjugation phase of biotransformation is at work. These two effects are in accordance and lead to an accumulation of 4-nitrocatechol (Fig. 1). This altered balance in *p*-nitrophenol metabolism is further supported by the decreased conjugation in starvation (Table 1). Decreased conjugation can be also the consequence of decreased UDP-glucuronic acid synthesis [37] and starvation itself also can result in UDP-glucuronic acid deficiency. However, our

experiments carried out in hepatocyte homogenates show that the decreased conjugation cannot be explained solely by the depletion of glycogen, or the non-availability of UDP-glucose as a 75% decrease of UDP-glucuronosyl transferase activity was observed also in the presence of exogenous UDP-glucuronic acid (Table 2). It is also noteworthy, that decreased conjugation is demonstrated in the case of phenolphthalein glucuronosyl transferase (Table 1), not regulated by cAMP-dependent phosphorylation [21]. However, the decreased rate of conjugation cannot be regarded as a prerequisite for accumulation of catechols and phenols. Acetone pretreatment results in an accumulation of unconjugated hydroxylated products without any decrease in *p*-nitrophenol glucuronosyl transferase activity. It is mentioned that sulfation plays a minor role in conjugation of *p*-nitrophenol; decreases of glucuronidation are responsible for changes of conjugation in starvation or in the presence of dibutyryl cAMP (G. Bánhegyi *et al.* unpublished observations).

Biological significance of these changes can be well interpreted if the main physiological function of cytochrome P-450 3a in rabbits is to participate in conversion of acetone to glucose as suggested by Koop *et al.* [23]. Thus, cytochrome P-450 3a is basically a gluconeogenic enzyme, and by this interpretation it is also conceivable that cAMP as a hunger signal does not inhibit an enzyme in the gluconeogenic sequence.

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