Biochemical Pharmacology, Vol. 37, No. 2, pp. 297–302, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

INDUCTION OF MICROSOMAL EPOXIDE HYDROLASE BY NITROSAMINES IN RAT LIVER

EFFECT ON MESSENGER RIBONUCLEIC ACIDS

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(*Received* 6 *May* 1987; *accepted* 21 *July* 1987)

Abstract—Nitrosomethylethylamine and nitrosomethylpropylamine were found to be more potent inducers of rat liver microsomal epoxide hydrolase (styrene oxide hydrolase) than nitrosodiethylamine or nitrosodimethylamine. The time course of induction following a single administration of nitrosodimethylethylamine, nitrosomethylpropylamine or nitrosodiethylamine each showed a delay of 24 hr during which enzyme activity was unaltered. After that time activity increased and reached a maximum at between 72 and 120 hr. Increased enzyme activity following NDEA was paralleled by changes in the content of epoxide hydrolase in microsomes as measured by Western blots. Nitrosamines caused an increase of mRNA for epoxide hydrolase which was detected by probing Northern blots with a [32]-P labelled epoxide hydrolase cDNA and by *in vitro* translation of polyadenylated mRNA. Both methods showed a maximal increase at 72 hr after nitrosodiethylamine treatment but a significant increase was also observed at 24 hr although at this time no increase in enzyme activity was apparent.

Pre-treatment of rats with certain xenobiotics increases the activity of the hepatic microsomal xenobiotic metabolising epoxide hydrolase (EC 3.3.2.3) (RmEH)‖ which is usually measured with styrene oxide or benzo(*a*)pyrene-4,5-oxide as substrate [1–3]. The range of compounds which induce this enzyme [1–4] is as diverse as that inducing cytochrome P-450 [4, 5] but the two systems appear to be under different genetic control [4]. Increased cytochrome P-450s result from increased levels of functionally active mRNA encoding specific isoenzymes [6–8]. The induction of epoxide hydrolase by phenobarbital [6, 9, 10] and 2-acetylaminofluorene [11] also results from an increase in translatable mRNA for the protein. Epoxide hydrolase activity can also be increased by nitrosamines such as nitrosodiethylamine (NDEA) [12]. It has not been established whether this effect results from direct interaction of NDEA (or its metabolites) with enzyme molecules or from increased enzyme synthesis. The effects of NDEA are of considerable interest since this compound has frequently been used during studies of experimental liver carcinogenesis [13–15]. In this study a comparison of the effects of a number of structurally related nitrosamines has been made and the time course of enzyme induction is described. NDEA was found to be without effect on enzyme activity *in vitro*. Instead it was found that an increase of enzyme activity *in vivo* was preceded by an increase of translatable mRNA for epoxide hydrolase following single administration of NDEA. A preliminary report of a part of this work has appeared [16].

MATERIALS AND METHODS

NDEA, NDMA, NMEA, NMPA, glucose-6-phosphate Na, glucose-6-phosphate dehydrogenase yeast Type VII and NADP were obtained from Sigma Chemical Co. (Poole, Dorset). [7(*n*)^{3}H]-Styrene oxide (161 mCi/mmol), [^{35}*S*]methionine (1280 Ci/mMol), [32]P-dCTP (4000 Ci/mol) and rabbit reticulocyte lysate (micrococcal nuclease treated) (N90) were purchased from Amersham International. Guanidinium thiocyanate and Na-*N*-lauroyl-sarcosine were obtained from Fluka AG (Buchs, Switzerland). Oligo(dT)-cellulose Type 7 was from P.L. Biochemicals (Milwaukee, WI). EcoR1, T4 DNA ligase, DNA polymerase and DNA polymerase Klenow fragment were purchased from Anglian Biotechnology Ltd (Essex, U.K.). DNA sequencing reagents and M13mp18 were from BRL/Gibco Biocult Ltd (Paisley, Scotland).

Male Wistar rats (200–250 g) were treated by single administration of nitrosamine, i.p., in saline, at the doses indicated and animals were sacrificed at various times thereafter. Phenobarbital administration was by single dose, 100 mg/kg, 16 hr prior to sacrifice. Microsomes were prepared from the pooled livers of at least two animals in high salt buffers [17] and epoxide hydrolase (styrene oxide hydrolase) activity determined [17] as described. The epoxide hydrolase

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‖ Abbreviations used: IPTG, isopropylthiogalactoside; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NMEA, *N*-nitroso-*N*-methyl-*N*-ethylamine; NMPA, *N*-nitroso-*N*-methyl-propylamine; RmEH, rat microsomal epoxide hydrolase; SDS, sodium dodecyl sulphate.

content of microsomes was also assessed by "Western" blotting. Microsomal proteins were resolved by SDS-polyacrylamide gel electrophoresis [18] prior to electrophoretic transfer to nitrocellulose filters (Schleicher and Schull) as described by Towbin *et al.* [19]. Epoxide hydrolase was visualized by immunostaining using a monospecific antibody to the enzyme [17] as described by Domin *et al.* [20] using 4-chloro-1-naphthol as substrate.

An immunoscreening technique was used to select clones containing epoxide hydrolase cDNA from a cDNA library from untreated rat liver in the expression vector λgt11 [21]. Phage were induced to synthesise fusion proteins by exposure to IPTG, the proteins transferred to nitrocellulose filters and plaques encoding epoxide hydrolase identified by immunostaining as described [20]. The putative RmEH clones were plaque purified and digested with EcoR1 to release cDNA inserts which were recovered by agarose gel electrophoresis. The insert from clone RmEH5 was approximately 900 kb and was subcloned into M13 [22] prior to sequencing by the dideoxy method [23]. This confirmed that RmEH5 encoded epoxide hydrolase since the sequence obtained corresponded exactly with that presented by Porter *et al.* [24] and extended from nucleotide 881 to 1750 of the published sequence. The insert was also subcloned into the EcoR1 site of pUC for the preparation of radiolabelled probes [25]. For this RmEH5 cDNA was excised with EcoR1, recovered by electrophoresis in low melting point agarose and heat denatured prior to incorporation of α-[32]-P-dCTP catalysed by Klenow fragment using random oligonucleotide primers [26].

Total cellular RNA was extracted by the guanidinium thiocyanate method [27] and in some cases was enriched for polyadenylated-mRNA by chromatography on oligo(dT)-cellulose [28]. Total RNA was electrophoresed on agarose gels (1%) in the presence of formaldehyde prior to Northern blot analysis as previously described [25]. Translational activity of polyadenylated mRNA (up to 1 μg/assay) was determined in mRNA dependent rabbit reticulocyte lysate system [29] in the presence of [^{35}S]methionine (40 μCi/assay) as directed by the suppliers. The proportion of radioactivity incorporated into epoxide hydrolase was determined essentially as described by Pickett and Lu [9].

RESULTS

Single administration of a range of structurally related nitrosamines caused a dose-dependent increase in the activity of microsomal epoxide hydrolase. In these studies, shown in Fig. 1A, NMEA and NMPA were found to be more effective than NDEA or NDMA and elicited a larger response at equivalent dose. The time courses of increased epoxide hydrolase activity following single administration of NMEA, NMPA or NDEA are shown in Fig. 1B. Following NDEA, enzyme activity reached a maximum by 72–120 hr and thereafter declined slowly. With each of the compounds tested enzyme activity was unaltered at 24 hr post-administration and a significant increase was only apparent at 48 hr (Fig. 1B). In these experiments this lag phase was observed whether NDEA was administered at 100 or 200 mg/kg.

To test whether NDEA or its metabolites increase epoxide hydrolase activity by direct interaction with the enzyme molecule, microsomes were pre-incubated with NDEA in the absence or presence of an NADPH-regenerating system [30]. Following this pretreatment, epoxide hydrolase activity was determined with styrene oxide as substrate and results are shown in Table 1. None of the pretreatments were found to affect enzyme activity.

Clear evidence that NDEA caused an increase of

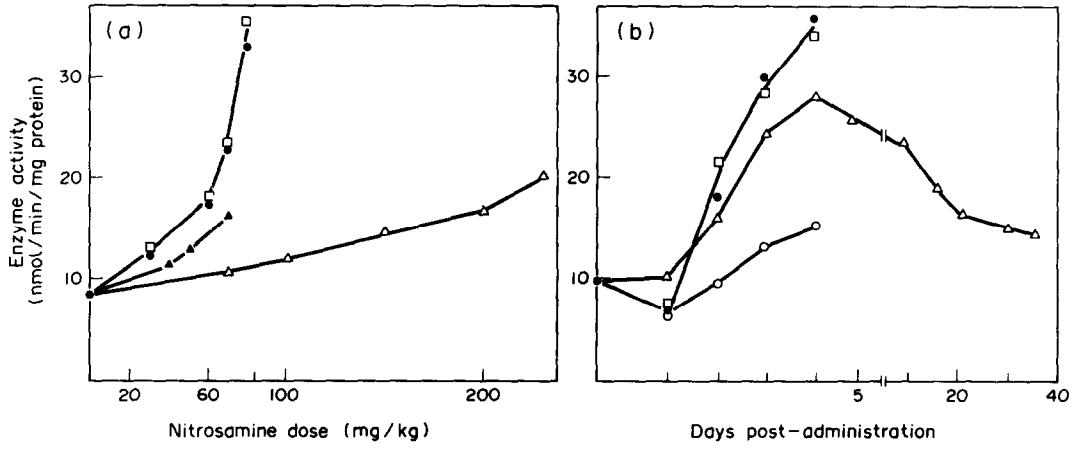

Fig. 1. Effect of nitrosamines on epoxide hydrolase activity of rat hepatic microsomal fractions. (a) Animals were treated with a single administration of either NMEA (●), NMPA (□), NDEA (△) or NDMA (▲) at the doses indicated and were sacrificed 120 hr later. Microsomes were prepared and styrene oxide hydrolase activity determined. The results show the mean of duplicate determinations which were within ±5%. (b) Animals were treated with a single administration of NMEA (●) (80 mg/kg), NMPA (□) (80 mg/kg), NDEA (○) (100 mg/kg) or NDEA (△) (200 mg/kg) and were sacrificed at the times indicated. Microsomes were prepared and styrene oxide hydrolase activity determined. Results are the mean of duplicate determination which were within ±5%.

Table 1. Effect of nitrosodiethylamine, *in vitro* on the activity of microsomal epoxide hydrolase

Addition	Enzyme activity (nmol/min/mg protein)
None	10.9 ± 0.7
NDEA (1 mM)	11.6 ± 2.3
NADPH regenerating system	9.5 ± 1.5
NDEA (1 mM) + NADPH regenerating system	9.6 ± 0.9

Microsomes were prepared from untreated animals and pre-incubated for 30 min either alone, in the presence of NDEA, in the presence of an NADPH regenerating system or in the presence of both NDEA and NADPH regenerating system. The conditions for pre-incubation and details of the NADPH system were as described [30] for the measurement of nitrosoalkylamine dealkylation activities. Aliquots were then taken for determination of styrene oxide hydrolase activity in Tris buffer pH 9.0.

the microsomal content of RmEH was provided by analysis of microsomes from treated animals by SDS-polyacrylamide gel electrophoresis and by Western blots. A polypeptide band which co-electrophoresed with purified RmEH (Fig. 2A) showed increased staining by Coomassie blue and by immunostaining following transfer to nitrocellulose filters (Fig. 2B). Changes in the microsomal content of the enzyme paralleled changes of enzyme activity and no increase of enzyme protein was observed before 24 hr of NDEA administration.

Other inducers of epoxide hydrolase exert their effects by increased synthesis of translatable mRNA encoding the enzyme. To examine the possibility that this is also the case for induction mediated by NDEA total hepatic RNA was extracted from untreated animals and animals treated with NDEA for various times. For comparison, RNA was also extracted from animals treated 16 hr previously with phenobarbital, a treatment producing maximal increase of mRNA for RmEH with this xenobiotic [6]. RNA was also extracted from animals treated 120 hr previously with NMEA. The RNA samples were electrophoresed in denaturing agarose gels and subjected to Northern blot analysis using [32]-P-labelled RmEH5 cDNA as probe. The results (Fig. 3) show that the probe hybridised with a single molecular species of about 2 kb in all samples and treatment with NDEA increased the intensity of the hybridisation signal. This signal reached a maximum in samples obtained from animals treated 72 hr prior to sacrifice and thereafter appeared to decline. Surprisingly the signal was significantly elevated by 17 hr. The hybridisation signal was also increased when animals received NMEA 120 hr prior to sacrifice or phenobarbital 16 hr prior to sacrifice (data not shown).

Polyadenylated-mRNA was isolated from some RNA samples and translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The incorporation of radioactivity into total protein and into RmEH was determined and results are shown in Table 2. The mRNA activity for epoxide hydrolase in samples of RNA from untreated animals constituted less than 0.1% of total mRNA activity. Messenger-RNA activity for epoxide hydrolase was increased by 5-fold when NDEA was administered 72 hr prior to sacrifice and a similar increase was found in samples from animals treated with phenobarbital (data not shown) [9]. mRNA activity for epoxide hydrolase was increased almost 3-fold at 24 hr post administration in accordance with the results of the Northern blot analysis.

Table 2. Effect of nitrosodiethylamine on the relative content of translatable polyadenylated RNA coding for epoxide hydrolase

Animal treatment	Epoxide hydrolase mRNA % total translation products	Fold increase
Untreated	0.066 ± 0.017	1.00
24 hours	0.181 ± 0.059	2.74
120 hours	0.378 ± 0.036	5.72

Total cellular polyadenylated RNA was isolated from untreated animals or those receiving a single administration of NDEA (200 mg/kg). Animals treated with NDEA were sacrificed at the times indicated after administration. The RNA samples were translated in a mRNA dependent rabbit reticulocyte lysate system in the presence of [^{35}S]methionine as directed by the suppliers. 2 μl aliquots were used to determine the total mRNA activity by precipitation with trichloracetic acid. Aliquots of the incubations containing between 0.126 and 3.599×10^6 dpm were immunoprecipitated with an antiserum specific for epoxide hydrolase in the presence of purified enzyme (20 μg). The antiserum was raised in rabbits using homogenous enzyme preparations as antigen (fraction CMB2 specific activity 735 nmol/min/mg [17]) and precipitated a single band of microsomal origin and which co-migrated with the purified enzyme [40]. The washed precipitates were redissolved and subjected to SDS–polyacrylamide gel electrophoresis [33]. The gels were stained with Coomassie blue and the band co-migrating with purified epoxide hydrolase was excised and treated with NCS (Amersham) solubilizer prior to determination of radioactivity. The results were corrected for background radioactivity (70 ± 8 dpm) which was determined in three bands above and below the specific band. The range of radioactivity in immunoprecipitates after correction was 547–2788 dpm. The results show the mean ± SD from three separate RNA preparations for each treatment.

DISCUSSION

Administration of a number of structurally related nitrosamines to rats caused a dose-dependent increase of microsomal epoxide hydrolase activity (Figs 1A and 1B). The magnitude of response to these compounds was in the order NMEA and NMPA > NMEA > NDEA. The comparison of relative effectiveness is necessarily qualitative since it was not possible to extend the dose–response curves beyond the points shown due to the toxicity of the compounds being used. Time courses of increased enzyme activity showed a distinct lag of approximately 24 hr before enzyme activity was altered. After treatment with NDEA, activity reached a maximum between 72 and 120 hr and then declined. The loss of activity was slow and, as found for 2-acetylaminofluorene [12, 31] basal levels were not reattained after 35 days.

It is possible that nitroso compounds act directly on epoxide hydrolase by allosteric activation of enzyme molecules. Various authors [32–34] have demonstrated activation *in vitro* by nitroso compounds of UDP-glucuronyl transferase, another microsomal xenobiotic-metabolising enzyme. Covalent modification of epoxide hydrolase is another possible mechanism of increased enzyme activity. The principle pathway of nitrosamine metabolism is by cytochrome P-450 catalysed oxidation and the resulting alpha-hydroxyalkylnitrosamine is unstable and spontaneously decays to an aldehyde and an alkylating group [35]. Covalent modification of epoxide hydrolase by alkylation may increase enzyme activity. However, incubation of microsomes with NDEA alone or under conditions in which the nitrosamine was metabolised, did not alter enzyme activity (Table 1) and thus direct activation seems unlikely. It is still possible that alkylation is involved *in vivo* if an alkylated enzyme had a longer half life than the unmodified enzyme since this would result in an increased microsomal content of the enzyme. The response to NDEA was indeed shown to include an increased microsomal content of the enzyme when microsomal samples were analysed by SDS–polyacrylamide gel electrophoresis and by Western blots (Figs 2A and 2B). Changes in the amount of RmEH protein correlated with changes in enzyme activity and no increased protein was observed until after a lag period of 24 hr.

The mechanism of increased RmEH is associated with increased levels of mRNA for the protein. RNA isolated from animals treated with NDEA showed increased levels of mRNA for epoxide hydrolase when measured by northern blots (Fig. 3) or by *in vitro* translation of polyadenylated-RNA (Table 2). The level of mRNA activity in samples from

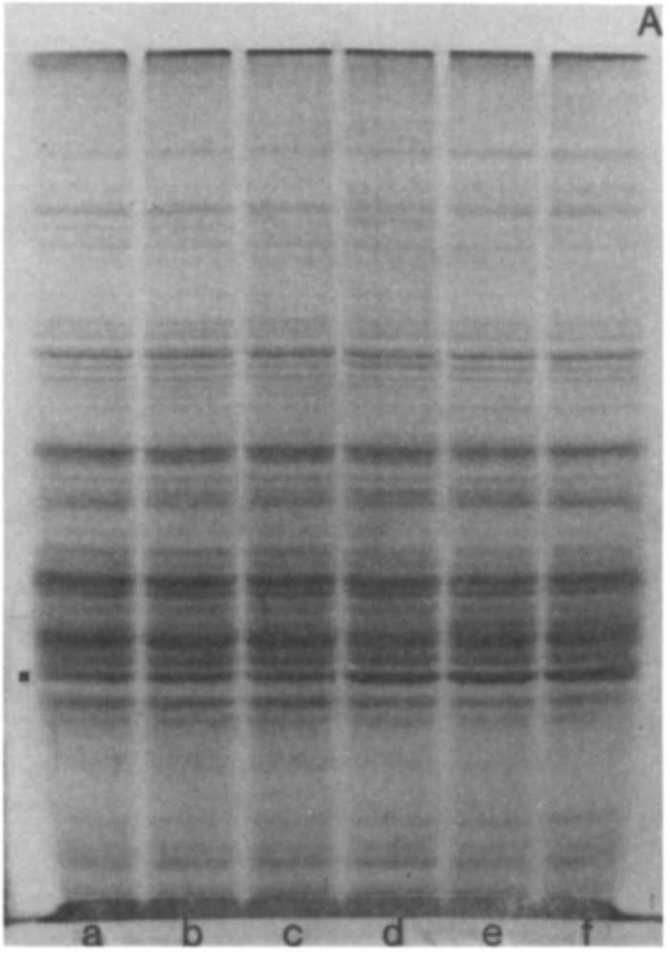

Fig. 2 (*continued on facing page*).

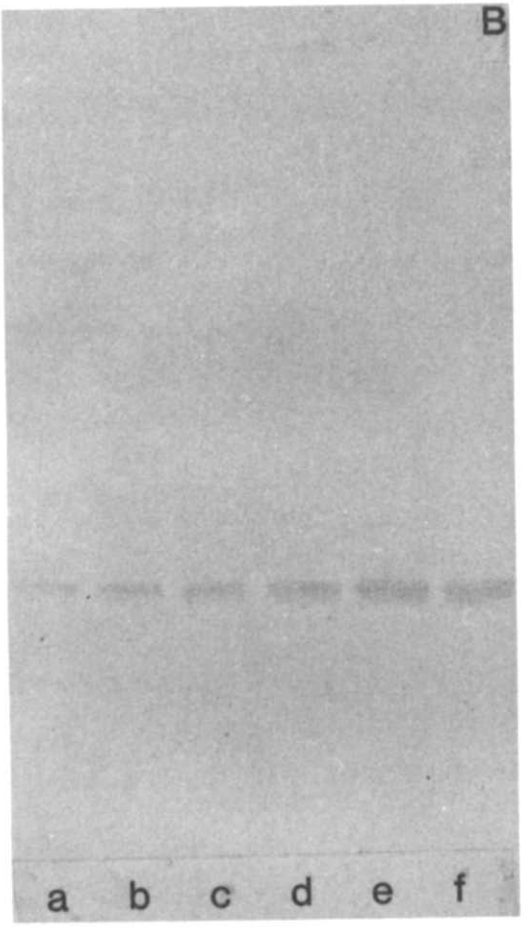

Fig. 2. SDS-polyacrylamide gel electrophoresis of rat hepatic microsomes from animals treated with NDEA. (A) Animals were treated with a single administration of NDEA (200 mg/kg) and sacrificed at various times thereafter. Hepatic microsomes (25 μg) were subjected to electrophoresis on 7.5% gels as described in the Methods and stained with Coomassie blue. The migration of purified RmEH is indicated (■). Samples were loaded: a, untreated; b, 17 hr; c, 24 hr; d, 36 hr; e, 72 hr; f, 120 hr where the times indicate the period between administration of NDEA and sacrifice. (B) Microsomal samples (12.5 μg) were loaded to identical SDS–polyacrylamide gels and electrophoresed as in (A) and transferred to nitrocellulose filters. The gels were exposed to monospecific anti-RmEH and immunoreactive polypeptides visualized using the immunoperoxidase method with 4-chloro-1-naphthol as substrate. The identity of samples was as in (A).

untreated animals in the *in vitro* system was in accord with findings of other authors [9–11]. Both methods showed that NDEA increased mRNA levels as early as 24 hr after administration and mRNA continued to increase reaching a maximum by 72 hr. The induction of other drug metabolising enzymes (cytochrome P-450, UDP-glucuronosyltransferase, glutathione transferase) also involves increased levels of protein specific mRNA but the mechanism of such responses has not been clearly defined. Induction by polycyclic aromatic hydrocarbons is mediated by a cytosolic receptor protein but there is no clear evidence for receptors for other classes of inducer (discussed in Ref. 36). Our finding that asymmetric nitrosamines are more efficient inducers of RmEH than similar symmetric compounds may indicate preference of binding to a putative receptor for these compounds. Alternatively the greater potency of the asymmetric compounds may relate to differences in the rates of their metabolism compared to the symmetric compounds [37, 38].

The results presented in this study indicate differences in the mechanism of induction of epoxide hydrolase mediated by nitrosamines as increase of enzyme activity following nitrosamine administration is considerably delayed. This is surprising since nitrosamines are subject to rapid metabolism and the liver plays a central role in this process [39]. The effects of phenobarbital and 2-acetylaminofluorene are more immediate and enzyme activity is significantly increased by 24 hours after administration [6, 31]. When phenobarbital is used as inducer, transcription of epoxide hydrolase genes is increased and reaches a peak value at 1 hr after drug administration [6]. Over the same period of time nuclear transcripts of epoxide hydrolase also increase. These changes precede an increase in cytoplasmic mRNA for epoxide hydrolase which peaks

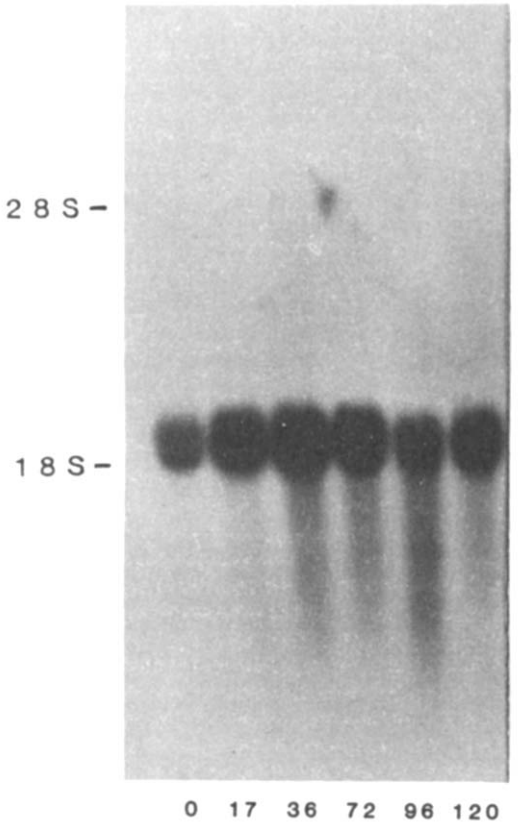

Fig. 3. Northern blot analysis of RNA samples isolated from the livers of NDEA treated rats. Animals were treated with single administration of NDEA (200 mg/kg) and sacrificed at various times thereafter. Total RNA was isolated and samples (15 μg) electrophoresed on a formaldehyde 1% gel. RNA was transferred to Hybond N and hybridised with [32]P-RmEH5 cDNA. Hybridisation was visualized by autoradiography. Rat rRNA (18S and 28S) were used as gel markers and their positions are indicated. The time after administration (hours) that samples were prepared is indicated.

at 16 hr and thereafter declines. Similar early increases in mRNA for epoxide hydrolase are observed following administration of 2-acetylaminofluorene [11]. For both compounds there is a correlation between functionally active levels of mRNA and enzyme activity. In the study reported here this correlation was not found. Thus while mRNA for epoxide hydrolase was markedly increased by 72 hr, at which time maximal enzyme activity was approached, mRNA was also elevated at 24 hr at which time no increase in enzyme activity is apparent. An explanation for this discrepancy awaits further experimentation but could result from delayed transfer of nuclear epoxide hydrolase mRNA to the cytoplasm or from translational inhibition.

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