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SHORT COMMUNICATIONS

Increase in acetyl CoA synthetase activity after phenobarbital treatment

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Administration of phenobarbital to rats produces microsomal enzyme induction [1] and enlargement of liver [2]. Prolonged treatment of mouse with phenobarbital causes increases of DNA and RNA contents [3]. Recent report shows that phenobarbital enhances activities of ornithine decarboxylase and *S*-adenosyl methionine decarboxylase, these enzymes participate in polyamine synthesis [4]. Since amino sugars as the constituents of glycoproteins are essential components of stroma of tissue, effects of phenobarbital on amino sugar metabolism were studied. The initial step of amino sugar synthesis is formation of glucosamine 6-P which is acetylated in the presence of acetyl CoA to *N*-acetyl glucosamine 6-P. This is finally converted via UDP-*N*-acetyl glucosamine and CMP-*N*-acetyl neuraminic acid to glycoprotein. This report deals with the effects of phenobarbital on the activities of glucosamine 6-P synthetase (L-glutamine:D-fructose-6-phosphate amidotransferase, EC 2.6.1.16), acetyl CoA synthetase (acetate: CoA ligase (AMP) EC 6.2.1.1), glucosamine 6-P acetylase (acetyl-CoA: 2-amino-2-deoxy-D-glucose-6-phosphate *N*-acetyltransferase, EC 2.3.1.4), glucosamine kinase (ATP: 2-amino-2-deoxy-D-glucose-6-phosphotransferase, EC 2.7.1.8) and acetyl glucosamine kinase (ATP: 2-acetamido-2-deoxy-D-glucose-6-phosphotransferase, EC 2.7.1.9).

Male Wistar rats, weighing 130–150 g were fed *ad lib.* with laboratory chow and maintained in a room at a constant temperature (23·5°) with 12 hr each of light (6·30 a.m.–6.30 p.m.) and darkness. All animals were sacrificed between 9 and 10 a.m. Phenobarbital sodium (100 mg/kg body wt per day), dissolved in 0·9% NaCl, was injected intraperitoneally between 9 and 10 a.m. Control animals received equivalent volume of 0·9% NaCl. The last dose was administered 24 hr prior to sacrifice. Livers from exsanguinated rats were homogenized with 10 vol of ice-cold 0·25 M sucrose containing 1 mM EDTA (pH 7·5) in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 20,000 *g* for 10 min, and the supernatant was centrifuged again at 105,000 *g* for 1 hr. The resulting supernatant was used immediately as enzyme solution.

Glucosamine 6-P synthetase activity was measured as follows [5]: 100 μmoles Tris–HCl buffer (pH 7·5), 20 μmoles L-glutamine, 20 μmoles D-fructose 6-P and the enzyme solution in a total vol of 1·0 ml. After incubation for 2 hr at 37°, the mixture was kept in a boiling water bath for 2 min. The protein precipitate was centrifuged off and 0·6 ml of the supernatant was analysed for glucosamine 6-P by the Elson–Morgan procedure [6]. D-Glucosamine was used as a standard. D-Glucosamine 6-P yields 85% of the optical density obtained with an equimolar solution of D-glucosamine.

Glucosamine 6-P acetylase was assayed as follows: the reaction system contained 100 μmoles Tris–HCl buffer (pH 8·0), 2 μmoles D-glucosamine 6-P (Sigma), 0·1 μmole CoA (Boehringer), 5 μmoles potassium acetate, 5 μmoles ATP, 5 μmoles $MgCl_2$, 1 μmole dithiothreitol, 30 μmoles diammonium phosphate, and the enzyme solution in a total vol of 1·0 ml. After 30 min incubation at 37°, the reaction was stopped by boiling for 2 min. After centrifugation, 0·1 ml of supernatant was withdrawn for acetyl glucosamine 6-P determination. Acetyl glucosamine 6-P yields the same optical density as is obtained with an equimolar solution of acetyl glucosamine [7]. The enzyme was also assayed as mentioned above except that partially purified acetyl CoA synthetase prepared from baker's yeast [8] (specific activity of alumina C_γ eluate fraction was 55 units/mg protein) was added and that half vol of enzyme solution was used.

Acetyl CoA synthetase activity assayed was as follows. Acetyl CoA formed yielded hydroxamic acid with hydroxylamine. Hydroxamic acid was measured colorimetrically with $FeCl_3$ reagent [9]. The reaction mixture contained in 1·0 ml 100 μmoles Tris–HCl buffer (pH 8·0), 0·1 μmole CoA, 5 μmoles potassium acetate, 5 μmoles ATP, 5 μmoles $MgCl_2$, 1 μmole dithiothreitol, 200 μmoles neutralized NH_2OH, 30 μmoles diammonium phosphate and the enzyme solution. The mixture was incubated for 60 min at 37°, then 1·5 ml of a $FeCl_3$ reagent was added which contains 10% $FeCl_3 \cdot 6H_2O$ and 3·3% trichloracetic acid in 0·66 *N* HCl. After centrifugation, the optical density at 540 nm was determined. Succino monohydroxamic acid was used as a standard [10].

Phosphorylation of glucosamine. The complete reaction mixture was as follows: 100 μmoles Tris–HCl buffer (pH 8·0), 10 μmoles D-glucosamine, 10 μmoles ATP, 20 μmoles $MgCl_2$, 1 μmole EDTA, 1 μmole dithiothreitol, 10 μmoles NaF, 100 μmoles KCl and the enzyme solution in a total vol of 1·0 ml. This was incubated at 37° for 30 min, and stopped by boiling for 2 min. After diluting the mixture with 4 ml of water, the denaturated protein was removed by centrifugation. Resulting supernatant was applied to Dowex 1-formate (200–400 mesh) column (diameter 0·6 cm, length 3·5 cm). Glucosamine was washed off by 10 ml of water and glucosamine 6-P was eluted by 5 ml of 0·01 M formic acid. After evaporation at 30°, the residue was dissolved in 0·6 ml of water, and glucosamine 6-P was assayed by the Elson–Morgan procedure [6].

Acetyl glucosamine kinase. The reaction mixture contained the following components in a final vol of 1·0 ml: 50 μmoles glycine–NaOH buffer (pH 9·4), 1 μmole *N*-acetyl D-glucosamine, 5 μmoles ATP, 10 μmoles $MgCl_2$, 10 μmoles NaF and the enzyme solution. After incubation at 37° for 30 min, the reaction was stopped by adding 0·2 ml of 5% $ZnSO_4 \cdot 7H_2O$ followed by 0·2 ml of 0·3 N $Ba(OH)_2$. After centrifugation to remove protein and acetyl glucosamine 6-P, an aliquot of the supernatant was taken for estimation of free *N*-acetyl glucosamine [7]. Disappearance of *N*-acetyl hexosamine corresponded to the amount phosphorylated.

Protein was determined colorimetrically according to the method of Lowry *et al.* [11].

The effects of phenobarbital on the activities on several enzymes concerning amino sugar metabolism namely glucosamine 6-P synthetase, glucosamine kinase, *N*-acetyl glucosamine kinase, acetyl CoA synthetase and glucosamine 6-P acetylase were studied. Assay conditions on glucosamine 6-P synthetase have already been reported [5]. The reactions of glucosamine kinase and acetyl glucosamine kinase proceeded linearly for 90 min and the reaction velocities were proportional to the amount of the enzymes up to 0·6 mg. The activities of glucosamine 6-P synthetase,

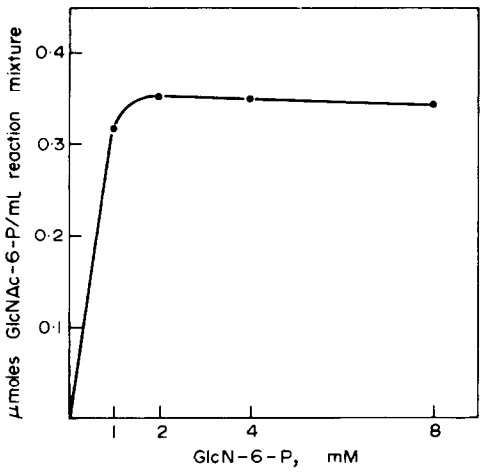

Fig. 1. Effect of glucosamine 6-P concentration on acetyl glucosamine 6-P formation by glucosamine 6-P acetylase (enzyme protein: 0·80 mg; time: 60 min).

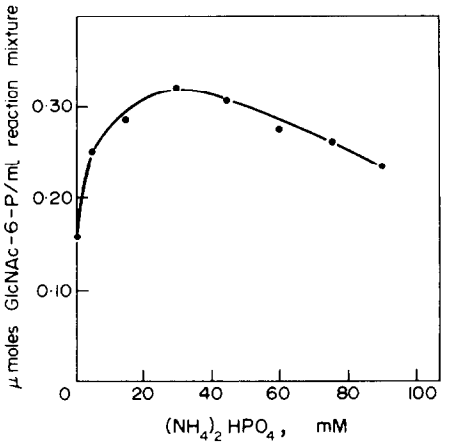

Fig. 2. Effect of ammonium phosphate concentration on the acetylation of glucosamine 6-P (enzyme protein: 0·80 mg; time: 60 min).

glucosamine kinase, and *N*-acetyl glucosamine kinase did not show any considerable changes after phenobarbital injection. The conditions of glucosamine 6-P acetylase assay were studied. Acetyl CoA synthesizing system was included in the reaction mixture instead of acetyl CoA since crude liver cytosol contained acetyl CoA synthetase [12]. Relationship between glucosamine 6-P acetylase activity and glucosamine 6-P concentration was shown in Fig. 1. To protect the deamination of glucosamine 6-P, it was necessary to include $(NH_4)_2HPO_4$ in the assay system, and its optimal concentration was 30 mM (Fig. 2). Glucosamine 6-P acetylase activity is proportional to time for 90 min and to the enzyme concentration up to 1·5 mg of protein. Phenobarbital injection until 3 days was found to enhance glucosamine 6-P acetylase activity (Table 1). The question was raised if acetyl CoA synthesized by rat liver extract was sufficient to supply acetyl group for glucosamine 6-P acetylase reaction. When partially purified acetyl CoA synthetase from yeast was added to the assay system of glucosamine 6-P acetylase, the reaction proceeded linearly for 80 min and up to 0·6 mg enzyme protein, and the enzyme activity in untreated animals was elevated 3·5 times. In such an assay condition, rat liver glucosamine 6-P acetylase activity did not change by phenobarbital treatment during 4 days. Thus, apparent increase of glucosamine 6-P acetylase activity by phenobarbital treatment may be caused by increased production of acetyl CoA. So, rat liver acetyl CoA synthetase activity was determined. The reaction proceeded linearly for 90 min and until 0·6 mg enzyme protein. Phenobarbital treatment during 4 days resulted in enhancement of acetyl CoA synthetase activity (Table 1). As was also shown in Table 1, the apparent increase of glucosamine 6-P acetylase activity paralleled the increase of acetyl CoA synthetase activity. To prove a direct relationship between protein synthesis and the increased activities of acetyl CoA synthetase, the effect of cycloheximide was examined. Cycloheximide (Wako Pure Chemicals, 1·5 mg/kg body wt) was administered with phenobarbital to the animals 2 and 3 days, and phenobarbital injection continued until 3 days. It produced marked inhibition upon acetyl CoA formation. The blocking effects of the antibiotic on enhancement of acetyl CoA synthetase activity during phenobarbital treatment seem to represent a *de novo* synthesis of the enzyme protein. Further study is required to clarify whether phenobarbital stimulates glycoprotein synthesis or not.

Many studies indicate that phenobarbital induces drug-metabolizing enzymes in the liver [1]. The enzymes are shown to be localized in liver microsome [1]. Recently, Japundžić *et. al.* reported that the drug induced mouse liver phosphoprotein phosphatase [13] located predominantly in the cytosol [14]. Remmer reported that acetylation of aminoantipyrine increased by phenobarbital treatment in dog [15]. Acetyl CoA synthetase reported here is located

Table 1. The activities of glucosamine 6-P acetylase and acetyl CoA synthetase after phenobarbital injection and effect of cycloheximide

	Controls	2 days	4 days	4 days (Cycloheximide injected rats)
	(mμmoles/mg protein per hr)			
Glucosamine 6-P acetylase	414 ± 51	555 ± 37	565 ± 76	396 ± 31
Acetyl CoA synthetase	526 ± 43	731 ± 30	750 ± 45	552 ± 63

Phenobarbital sodium (100 mg/kg) was administered intraperitoneally at 0, 1, 2 and 3 days to groups of 3 rats each. They were sacrificed at 2 and 4 days. Control animals received saline. Cycloheximide (1·5 mg/kg) was injected intraperitoneally into rats at 2 and 3 days. The activities of the enzymes were determined as described in the text. Results are expressed as mean ± S.D. of 3 rats in each group.

in cytosol of rat liver [12]. The induction of the enzyme by phenobarbital injection plays an important role in acetylation process in drug metabolism.

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Malathion A and B esterases of mouse liver—II Effect of EPN *in vitro* and *in vivo*

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EPN [*O*-ethyl *O*-*p*-nitrophenyl phenylphosphorothionate] potentiates the toxicity of malathion [*S*-(1,2-dicarbethoxyethyl) *O,O*-dimethyl phosphorodithioate] in mammals [1]. This synergism is attributed to the inhibition by EPN, via its oxygen analogue EPNO formed by oxidative desulphation in the liver, of non-specific carboxylesterases which normally detoxify malathion [2, 3]. Malaoxon, the active oxygen analogue of malathion would thus accumulate resulting in increased toxicity. Although the above sequence of events is a satisfactory working hypothesis to explain EPN–malathion synergism, some anomalies have been pointed out [4–6]. More recently, another inconsistency has been reported by Cohen and Murphy [7]. Whereas one would expect that the ability of tissues to hydrolyse malathion would be considerably reduced by the administration of EPN, the inhibition of this activity seems to be the lowest. Cohen and Murphy found that the inhibition of the esterase activities in mouse liver with diethyl succinate, methyl butyrate and triacetin as substrates was 90, 82 and 70%, respectively, while that with malathion itself was only 30%.

In a previous communication [8] we reported that there are two types of esterases in mouse liver which hydrolyse malathion. We had named these malathion A and B esterases. The effect of EPN *in vitro* and *in vivo* on these esterases is reported in this communication.

MATERIALS AND METHODS

The materials and methods used were the same as in the previous paper [8]. For injections of EPN, the required amount/kg was dissolved in 0·4 ml ethanol and diluted to 4 ml with propylene glycol. Appropriate volumes were injected intraperitoneally into mice and the animals were sacrificed after 1 hr. Malathion A and B esterase activities were determined as described in the previous communication. Esterase activity with phenyl acetate (PA) as substrate was determined at 20° according to the method given by Ramachandran and Ågren [9].

RESULTS

In vitro EPN at 10^{-5} M had only a weak inhibitory effect on malathion A and B esterases, the extent of inhibition being 17 and 13% respectively. EPNO had no effect

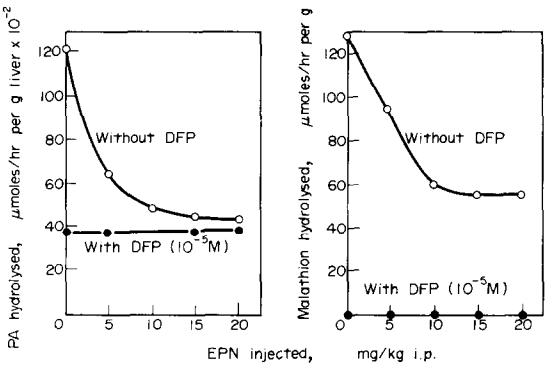

Fig. 1. Effect of injected EPN on mouse liver esterase activity with phenyl acetate (*left*) and malathion (*right*) as substrates. Conditions: for phenyl acetate, 0·067 M phosphate buffer pH 7·2 with 0·1% Triton X-100, phenyl acetate 10^{-2} M, 20°; for malathion B esterase 0·05 M Tris–HCl buffer pH 7·6 with 0·1% Triton X-100, malathion 10^{-3} M, 37°. In both cases duplicates were run simultaneously with homogenates which had been preincubated with 10^{-5} M DFP for 30 min at 0°. Each point is the average of 5–10 separate experiments. The residual malathion B esterase activity was completely inhibited by DFP (see the line at the base of figure on right).