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DISTRIBUTION, INDUCTION AND PURIFICATION OF A MONOOXYGENASE CATALYZING SULPHOXIDATION OF DRUGS

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Abstract—The enzyme, a monooxygenase, catalyzing the sulphoxidation of ethionamide (2-ethyl thioisonicotinamide) to ethionamide sulphoxide is located primarily in the liver and serum of guinea pigs. Administration of ethionamide enhances the activity of the enzyme; the enhancement in activity is due to increased synthesis of the enzyme. This oxygenase is not specific to ethionamide because compounds like chlorpromazine and thioridazine are also oxidized by the enzyme. However, administration of chlorpromazine itself does not induce the enzyme. The enzymes in various tissues (liver, pancreas, spleen, kidney and lungs) and serum of guinea pigs have been purified to homogeneity, as evidenced by electrophoretic and antigenic criteria. The comparison of properties of all these enzyme preparations reveals that the enzymes are identical. There is no apparent involvement of cytochrome P-450 in the sulphoxidation reaction.

Ethionamide (2-ethyl thioisonicotinamide) is an active tuberculostatic second line drug, which plays an important role in the retreatment of pulmonary tuberculosis, resistant to one or more of the classical drugs. The drug possesses very high activity and relatively low toxicity, thus offering good scope for the treatment of tuberculosis, especially in cases of infection caused by organisms resistant to primary drugs like isoniazid or streptomycin. Ethionamide is readily converted into its sulphoxide on administration to guinea pigs [1]; ethionamide sulphoxide has also been identified as a metabolite in man and rabbits [2–5]. The enzyme, an oxygenase, responsible for the conversion of ethionamide to ethionamide sulphoxide has been shown to be localized in the microsomal fraction of liver, in guinea pigs [6]. The enzyme is a flavoprotein, containing nonhaem iron and requires NADPH for its activity; the sulphoxidation is mediated through the formation of superoxide anions [6, 7]. Since ethionamide sulphoxide shows more *in vitro* antituberculous activity when tested against *Mycobacterium tuberculosis* H37Rv than ethionamide itself [1], the metabolic conversion may have significant influence on the action of the drug *in vivo*.

In this communication, we report the distribution of the oxygenase catalyzing the sulphoxidation, in various tissues and the serum of guinea pigs and the effect of ethionamide administration on the status of these enzymes. The enzymes localized in various tissues have been purified to homogeneity and their properties have been compared.

MATERIALS AND METHODS

Ethionamide (Trescatyl) was obtained from May & Baker Ltd, Dagenham, Essex, U.K.; chlorpromazine from May & Baker (India) Ltd, Bombay, India and mellaryl (thioridazine) from Sandoz (India) Ltd, Bombay, India. All these compounds were recrystallized before use. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Noble agar was from Difco Laboratories, Detroit, Michigan, U.S.A. The reagents for gel electrophoresis were from Eastman Kodak Co., Rochester, N.Y., U.S.A. Cycloheximide, actinomycin D, all of the marker enzymes (alcohol dehydrogenase, β-galactosidase, catalase etc.), cofactors and other biochemicals (Tris, Hepes† etc.) were from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Carbon monoxide differential spectra were recorded using Cary Model L2 spectrophotometer.

Polyacrylamide gel electrophoresis was done by the method of Ornstein and Davis [8] using Tris–glycine buffer, pH 8.3 or β-alanine–acetic acid buffer, pH 4.3, on 7.5% (w/v) polyacrylamide gels. The gels were stained with Amido Schwartz. SDS (sodium dodecyl sulphate)–polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [9].

The approximate molecular weight of the enzyme was determined by gel filtration [10] and SDS–polyacrylamide gel electrophoresis [9].

Preparation of antiserum against the enzyme and the techniques of immunodiffusion and immunoelectrophoresis were carried out as described earlier [6]. The pure enzyme preparation from liver was used as antigen, in Freund's complete adjuvant to raise the antienzyme serum from rabbits.

The protein content was determined by the biuret method [11] in crude extracts or by the method of Lowry *et al.* [12] in purified preparations. Bovine serum albumin fraction V was used as standard.

Enzyme assays. The enzymic sulphoxidation of ethionamide was assayed as described by Prema and Gopinathan [6]. A typical reaction mixture contained, in a final volume of 1.5 ml, 0.2 μmole NADPH, 0.36 μmole substrate, 0.02 M potassium phosphate buffer, pH 7.4, and varying amounts of tissue extracts. Incubations were carried out at 38° for

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† Abbreviations: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*′-yl) ethanesulphonic acid; SDS, sodium dodecyl sulphate.

90 min under aerobic conditions. At the end of the reaction, the unreacted substate and the product formed were extracted from the assay mixture into chloroform after saturation with ammonium sulphate and reextracted into dilute hydrochloric acid. The extinction of the samples at 315 nm (for ethionamide) and at 395 nm (for ethionamide sulphoxide) was recorded. A standard curve was prepared by using a mixture of authentic samples of these compounds.

The assay system was similar when chlorpromazine was used as a substrate. Chlorpromazine and chlorpromazine sulphoxide were estimated as described by Salzman and Brodie [13]; they were extracted with heptane containing 1.5% isoamyl alcohol and the heptane phase was taken into 0.1 N hydrochloric acid. The extinction of the aqueous phase was measured at 255 nm and 270 nm. A standard curve was prepared using a mixture of authentic samples of these compounds. The product of the enzyme reaction was identified as chlorpromazine sulphoxide by chromatography of the samples after solvent extraction on thin layer Silica gels and by comparison of their R_f values and u.v. absorption spectrum, with those of authentic samples.

A unit of enzyme activity is defined as nmoles of product formed/min under the conditions of the assay and the specific activity is expressed as units/mg of protein.

Treatment of animals and preparation of tissue homogenates. Male guinea pigs (700–800 g), random bred, were used in all experiments. The animals were fed *ad lib.* on stock diet obtained from Hindustan Lever Ltd, Bombay, India. Ethionamide was dissolved in 10% citric acid containing sucrose and was given orally at a dose of 140 mg/kg. Controls were treated with a solution containing only citric acid and sucrose. Cycloheximide (10 mg/kg) and actinomycin D (400 μg/kg) were injected intraperitoneally.

The blood samples were collected by cardiac puncture, for the preparation of serum. The tissues (liver, kidney, spleen, pancreas and lungs) were removed and homogenized in an MSE Nelco homogenizer for 2 min with 1.15% potassium chloride (2 ml/g wet tissue). The homogenates were centrifuged at 9000 *g* for 20 min and the supernatants were used for the determination of enzyme activity.

Purification of the enzyme from the different tissue homogenates and serum was carried out using the same steps and sequence as those described for the purification of the liver enzyme [6].

RESULTS

Distribution of the enzyme. The activity of the enzyme catalyzing the conversion of ethionamide to its sulphoxide was examined in various tissue homogenates. The highest sp. act. (nmoles of product formed/min/mg protein) of 0.09 was found in the liver followed by pancreas (0.035), spleen, kidney (0.020) and lung (0.009); the serum also showed a fairly good activity (0.06).

The enzyme acted on other substrates like chlorpromazine and thioridazine. Chlorpromazine was readily converted to chlorpromazine sulphoxide, but the sp. act. of the enzyme was lower compared to ethionamide sulphoxidation (only 10 per cent). The action on thioridazine was demonstrated indirectly by using it as an inhibitor for ethionamide sulphoxidation (since there was not any ready assay for thioridazine sulphoxide). Thus, at equimolar concentrations, thioridazine inhibited the sulphoxidation of ethionamide by 62 per cent; when the concentration of thioridazine was increased to twice or more than the molar concentration of ethionamide, there was complete inhibition.

Induction of the enzyme. Administration of ethionamide enhanced the levels of the enzyme, in guinea pigs (Fig. 1). In the liver, there was a 7–10-fold increase in sp. act. of the enzyme reaching the maximum in 24 hr which came down to almost normal

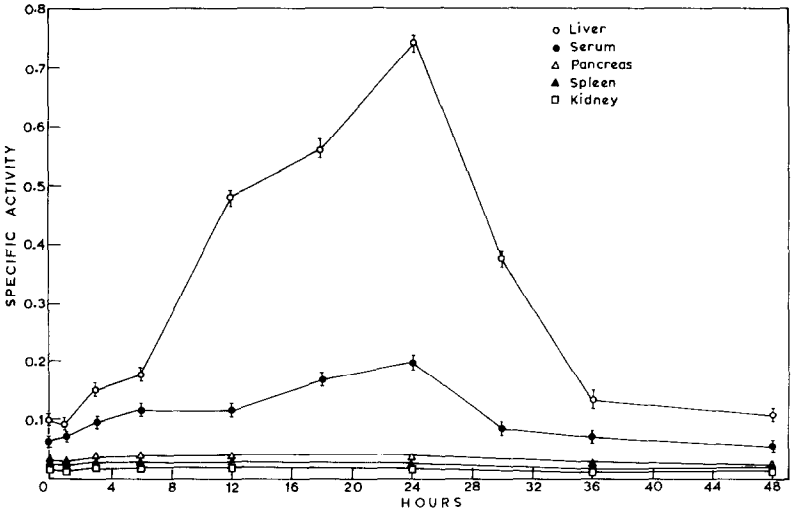

Fig. 1. Induction of the enzyme. Ethionamide was administered orally, at a dose of 140 mg/kg, and the animals were sacrificed at different time intervals. The tissue homogenates and sera were prepared and the enzyme assays were carried out as described under Materials and Methods. Enzyme sp. act.; O—O, liver; ●—●, serum; Δ—Δ, pancreas. ▲—▲, spleen and □—□, kidney.

Table 1. Sulphoxidation of ethionamide and chlorpromazine by the liver enzyme

Treatment of animals	Sp. act. of the enzyme (nmoles of product formed/min/mg protein)*	
	Ethionamide	Chlorpromazine
None (control)	0.092 ± 0.005	0.010 ± 0.001
Ethionamide	0.748 ± 0.012	0.078 ± 0.045
Chlorpromazine	0.090 ± 0.005	0.010 ± 0.001

* The values are expressed as mean ± S.D.

The animals (5 in each group) were sacrificed 24 hr after the administration of the drug and the livers were removed immediately and processed for enzyme activity. Ethionamide was administered orally at a dose of 140 mg/kg as a solution in 10% citric acid, containing sucrose. The animals in the control group were given 10% citric acid containing sucrose. Chlorpromazine was injected i.p. (50 mg/kg).

The details of the enzyme assay for sulphoxidation of ethionamide and chlorpromazine, are given under Materials and Methods.

levels in 36 hr. In the other tissues, the levels of enzyme activity did not show any change. The activity of the serum enzyme increased and came down to normal levels as in the liver; the highest sp. act. attained, however, was still much lower than that of the liver.

Ethionamide treatment also enhanced the enzyme activity with chlorpromazine (Table 1). Nevertheless, the administration of chlorpromazine itself to the animals, did not lead to increased levels of enzyme activity. Thus, although chlorpromazine can serve as a substrate for the enzyme it does not induce the synthesis of the enzyme.

In order to see whether the enhancement of enzyme activity after ethionamide administration was due to the induction of enzyme synthesis or only to the activation of previously existing enzyme, the effect of actinomycin D (transcription inhibitor) and cycloheximide (translation inhibitor) on the enzyme levels was studied. The results are presented in Table 2.

On administration of cycloheximide or actinomycin D, the enhanced levels of activity decreased to below those of the controls. Even in the case of control animals (not given ethionamide), actinomycin D and cycloheximide administration led to large decrease in enzyme activity.

Purification and properties of the oxygenase from various tissues. The enzyme was purified from the various tissues and serum of guinea pigs. The purification steps used were the same from all sources as standardized for the enzyme from liver and they were as follows (carried out in sequence, starting from the 9000 *g* supernatants of the tissue homogenates or serum): ammonium sulphate precipitation (40–75 per cent saturation), adsorption and elution from calcium phosphate gel, adsorption and elution from DEAE cellulose, a second ammonium sulphate precipitation at alkaline pH and gel filtration on Sephadex G-200 column (for details, see ref. 6). The enzyme preparations from all the tissues and serum showed the same pattern of purification with a total recovery ranging from 28 to 35 per cent.

The purified enzyme preparations from all the tissues showed a single band on polyacrylamide gel electrophoresis (both at pH 8.3 and 4.5). The immunodiffusion tests with the antiserum (prepared against the pure enzyme from liver) using the Ouchterlony double diffusion technique showed a single precipitin band (Fig. 2a and b). Immunoelectrophoresis also revealed the presence of only one band (Fig. 3a and b) in all cases. These results clearly indicate that the enzyme preparations were immunologically homogeneous and indistinguishable.

The general properties of the enzyme were also

Table 2. Effect of actinomycin D and cycloheximide on the induction of the enzyme

Groups	Treatment	Sp. act. (units/mg of protein)*	Percentage inhibition
Control	None	0.092 ± 0.005	—
	Actinomycin D	0.021 ± 0.005	77
	Cycloheximide	0.015 ± 0.002	83
Experimental	Ethionamide	0.720 ± 0.021	—
	Ethionamide + actinomycin D†	0.006 ± 0.0003	96¶
	Ethionamide + actinomycin D‡	0.002 ± 0.0001	98¶
	Ethionamide + cycloheximide§	0.001 ± 0.0002	99¶
	Ethionamide + cycloheximide‖	0.001 ± 0.0003	99¶

The animals were fasted for 3 hr prior to the administration of actinomycin D or cycloheximide. Each group contained 5 animals. In the control groups, actinomycin D (400 μg/kg) or cycloheximide was administered as a single dose, intraperitoneally. Ethionamide was administered orally at a dose of 140 mg/kg. The animals were sacrificed 24 hr after drug administration and the liver was immediately processed for enzyme activity.

* Unit = nmoles of product formed/min at 38°. The values are expressed as mean ± S.D.

† Actinomycin D (400 μg/kg) was injected i.p. 15 min prior to the administration of ethionamide.

‡ Actinomycin D (400 μg/kg) was given in 2 equal doses, 15 min prior to and 6 hr after the administration of ethionamide.

§ Cycloheximide (10 mg/kg) was administered 15 min prior to the administration of ethionamide.

‖ Cycloheximide (10 mg/kg) was given in 2 equal doses, 15 min prior to and 6 hr after the administration of ethionamide.

¶ The extent of inhibition is even more pronounced if calculated on the basis of induced enzyme levels.

studied and are listed in Table 3. Irrespective of the source of enzyme, all of them exhibited the same general properties and sensitivity to the enzyme inhibitors.

DISCUSSION

The induction of liver microsomal enzymes is important pharmacologically as it leads to an accelerated biotransformation of drugs *in vivo* and thereby alters the duration and intensity of drug action in animals. When the metabolite has a potency comparable with or greater than the drug, enzyme induction may intensify the effects of the drug by accelerating the production of the metabolite. Several drugs are

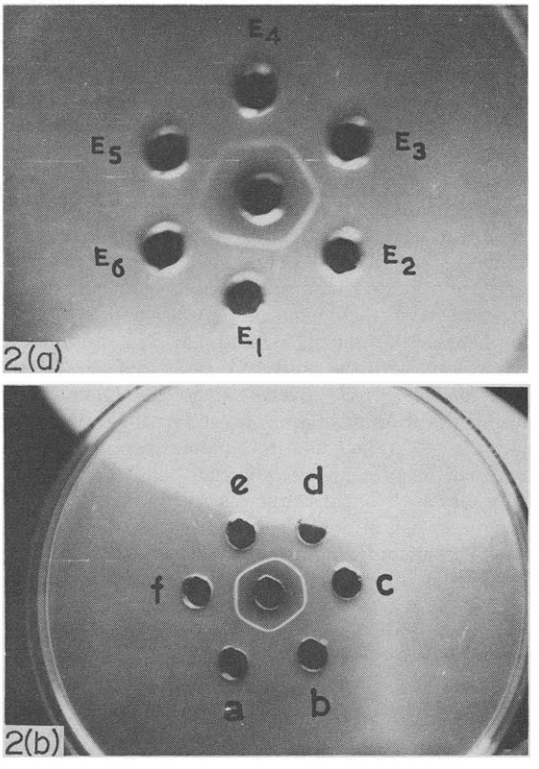

Fig. 2. Immunodiffusion patterns of the enzyme. The immunodiffusion was carried out by the Ouchterlony double diffusion method. Antiserum against purified enzyme (from liver), was prepared from rabbits. Subcutaneous injections of 2.5 mg protein in Freund's complete adjuvant were given at weekly intervals for 3 weeks and the animals were bled one week after the third injection, for the preparation of antienzyme serum. The immunodiffusion plates were washed (after keeping for 72 hr in the cold) with 0.9% NaCl, followed by water and then stained with amidoblack; the plates were destained by washing with methanol–acetic acid–water (2:3:35). (a) Varying concentration of the purified enzyme from liver. The central well contained the antiserum; the peripheral wells E1, E2, E3, E4, E5 and E6 contained 250, 125, 63, 32, 16 and 8 μg respectively, of the purified enzyme from liver. (b) Immunodiffusion against purified enzymes prepared from the different tissues and serum. The central well contained the antiserum. 50 μg of purified enzyme preparations were used in the peripheral wells. The wells a, b, c, d, e and f contained purified enzymes from liver, kidney, spleen, lungs, pancreas and serum.

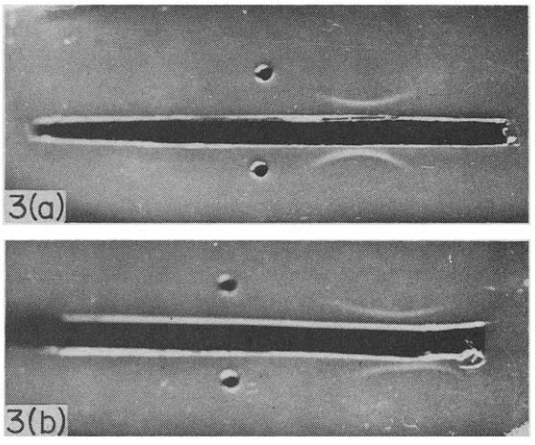

Fig. 3. Immunoelectrophoresis of the purified enzyme preparations. Immunoelectrophoresis at pH 8.6 was carried out in agar gel (1.5%, w/v) on microscope slides. The plates were stained after completion of the immunodiffusion, as described under Fig. 2. The wells contained the enzyme and the central trough contained the antiserum prepared against the pure enzyme from liver. (a) top, kidney enzyme; bottom, liver enzyme. (b) top, liver enzyme; bottom, pancreas enzyme.

Table 3. Properties of the enzyme from guinea pig liver, pancreas, kidney, spleen, lungs and serum

Molecular weight*	85,000
Homogeneity	
(a) polyacrylamide gel electrophoresis	single band
(b) immunodiffusion	single band
(c) immunoelectrophoresis	single band
Optimum pH†	7.4
Optimum temperature	38–39°
Thermal stability‡	50°
K_m value for ethionamide	5.9×10^{-5} M
Cytochrome P-450§	absent

* The molecular weight of the enzyme from liver was determined by SDS-polyacrylamide gel electrophoresis, gel filtration on a calibrated column of sephadex G-200, sucrose gradient centrifugation and analytical ultracentrifugation (sedimentation velocity method) as given in ref. [6]. The mol. wt of the other enzyme preparations was determined only by SDS-polyacrylamide gel electrophoresis and gel filtration on sephadex G-200. The markers used for gel filtration were catalase (250,000), alcohol dehydrogenase (150,000), bovine serum albumin (68,000), ovalbumin (45,000) and horse radish peroxidase (40,000). For gel electrophoresis, β-galactosidase (130,000), ovalbumin (45,000), pepsin (35,000), lysozyme (14,300) and cytochrome (11,700) were used as mol. wt markers.

† The optimum pH for the enzyme activity was determined in different buffer systems, viz., Tris–HCl (pH 7–9), Tris–maleate (pH 5.5–8.5), Hepes (pH 6.8–8.2) and potassium phosphate (pH 6.0–8.0).

‡ The enzyme was maintained for 1 min at various temperatures and the given value represents 50% inactivation. At temperatures of 55° and above, there was complete inactivation of the enzyme within 1 min.

§ The presence of cytochrome P-450 was checked by the carbon monoxide differential spectrum of the enzyme in a Cary recording spectrophotometer. The enzyme preparations were not sensitive to inhibition by SKF 525A (a known inhibitor of microsomal oxidases).

metabolized by liver microsomes through the mixed-function oxidase system. Often, the drugs activate or enhance the levels of the components of the mixed-function oxidase system which actually make up the membrane constituents of the endoplasmic reticulum. Thus, hypertrophy of the endoplasmic reticulum may be taken as a response of the animal liver towards increasing its capacity to metabolize the drug. Many groups of workers have attempted to correlate the ability of drugs to increase the levels of NADPH-cytochrome *c* reductase and cytochrome P-450 in microsomes, with their ability to enhance the metabolism of various other drugs [18–21].

The results presented in this paper indicate that on administration of ethionamide to guinea pigs, there was induction of the enzyme involved in the sulphoxidation of the drug. The induction of enzyme synthesis was found only in the case of liver. A small increase in the activity of the serum enzyme may be due to the release of the enzyme from its site of synthesis, which is presumably the liver. This enzyme has already been shown to be a monooxygenase containing 2 moles of FAD and 1 g atom of iron per mole and which required NADPH and molecular oxygen for activity [6]. Further, the mechanism of sulphoxidation has been elucidated which involves the participation of superoxide anions [7]. Analysing the substrate specificity of the enzyme, it is apparent that the enzyme is not specific to ethionamide oxidation and may act on other substrates (drugs) like chlorpromazine and thioridazine which can undergo biological sulphoxidation. Oxidation of chlorpromazine to its sulphoxide has been reported previously [13, 22], but the enzyme system responsible has not been characterized so far. Unlike ethionamide, chlorpromazine administration to the animals, however, did not induce the enzyme synthesis.

The enzymes distributed in the various tissues and serum have been purified to a homogeneous state using the same procedure as standardized for the liver [6]. It is clear from the results presented that they all possessed similar biochemical properties and were immunologically indistinguishable. From this and the enzyme induction data, it is reasonable to conclude that the liver is the primary site for the synthesis of the enzyme which is subsequently distributed in various other tissues. The induced enzyme preparations from liver and serum showed the presence of a small amount of protein separating from the bulk of enzyme at the Sephadex gel filtration stage in purification, but possessing the enzyme activity. This protein had a molecular weight of 110,000 (i.e., approximately 25,000 more than the normal enzyme) in both cases (unpublished results of the authors). Even these fractions did not reveal the presence of cytochrome P-450 and it is not established whether the apparent increase in molecular weight is due to the attachment of any other protein factors. However, this species of protein constituted only 10% of the total enzyme.

The sulphoxidation reaction may be of special significance with certain drugs like ethionamide which is readily metabolized to the sulphoxide on administration to the experimental animals and man [2–5, 7]. Ethionamide sulphoxide appears to be more potent *in vitro* in its antitubercular action than the parental compound [1] and hence the metabolic conversion may lead to a higher degree of activity of the drug *in vivo*.

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