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A microsomal amidase which cleaves chloramphenicol*

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ONE OF THE major excretory products of the antibiotic chloramphenicol is chloramphenicol base [D(−)-*threo*-1-(*p*-nitrophenyl)-2-aminopropane-1,3-diol], a metabolite which has lost a dichloroacetyl group and which has no antibacterial activity.[1,2] We report here the presence of an enzyme in the microsomal fraction of mouse liver which cleaves chloramphenicol, liberating dichloroacetate.

Chloramphenicol (dichloroacetyl-2-^{14}C), with a specific activity of 8·5 mCi/m-mole, was purchased from Calatomic, Los Angeles, Calif. The sample contained no detectable radioactive impurities. Microsomes and mitochondria were prepared from the livers of female DBA/2 mice. The animals, weighing about 25 g, were killed by cervical dislocation; the livers were excised, cooled and homogenized in 3 vol. of 0·25 M sucrose. After centrifuging the homogenate for 5 min at 900 *g*, the supernatant fraction was removed and centrifuged at 9000 *g* for 15 min to obtain the mitochondrial fraction, which was washed twice by centrifugation in 0·25 M sucrose. Microsomes were prepared from the 9000 *g* supernatant fraction by centrifuging at 100,000 *g* for 45 min and were washed once in 0·25 M sucrose.

The standard reaction system for chloramphenicol cleavage contained the following: microsomes equivalent to 40 mg liver; sodium glycinate buffer (pH 9·0), 12 μmoles; ^{14}C-chloramphenicol, 87 nmoles; and water in a total volume of 175 μl. The reaction was initiated by addition of the microsomal preparation and was stopped by streaking a 50-μl portion on paper strips. Incubation of the preparation was for 30 min at 37°. Substrate and metabolites were separated by paper chromatography with isopropanol–NH_3–H_2O (85:5:15, by vol.) and were measured with a Packard 7201 radiochromatogram scanner. The R_f-value for chloramphenicol was 0·96 and that for the microsomal metabolite was 0·67.

The electrophoresis experiments involved a buffer of 10% pyridine adjusted to pH 7 with glacial acetic acid and a Savant flat-plate apparatus. A potential of 40 V/cm was applied for 1 hr. Mass spectra were obtained with a Hitachi high-resolution, double-focusing mass spectrometer (RMU-6-D-3). Protein was determined by the method of Lowry *et al.*[3]

The pH optimum for the reaction was 9·0 with sodium glycinate as buffer. Higher pH values of this buffer were less effective, as were pH values 6·2–7·6 with phosphate buffer and pH values 6·9–8·9 with Tris–chloride. No detectable chemical hydrolysis was observed up to pH 9·8. Within the limits employed, the reaction was dependent upon the amount of microsomes present and the time of incubation and followed Michaelis–Menten kinetics (Fig. 1). K_m values for two separate determinations were 0·77 and 0·70 mM. Concentrations of NADPH up to 5 mM had no effect on the rate of reaction.

On the basis of protein added to the reaction system, microsomes had more than six times the activity found in either mitochondria or the 100,000 *g* supernatant fraction. Addition of the 100,000 *g* supernatant fraction to the system containing microsomes resulted in the further metabolism of the microsomal product, with new unidentified peaks appearing at R_f-values of 0·03, 0·10 and 0·47.

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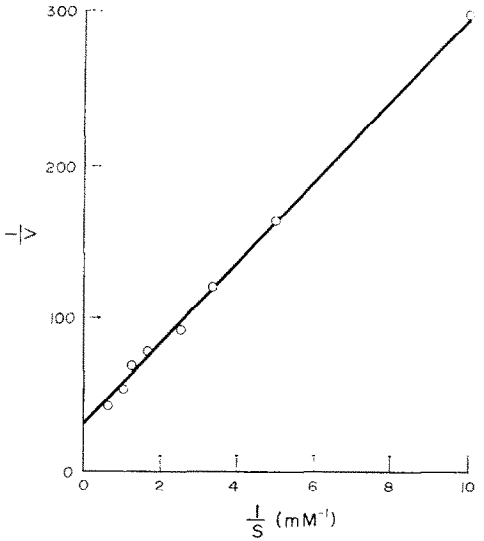

FIG. 1. Determination of the Michaelis constant for the enzyme cleaving chloramphenicol. The standard assay was used, except that the concentration of ^{14}C-chloramphenicol was varied as indicated. The values for the ordinate are reciprocals of the micromoles of ^{14}C-dichloroacetate liberated.

A sample of ^{14}C-chloramphenicol chemically hydrolyzed in 1 N KOH for 2 hr contained a single radioactive component which was anionic and which migrated the same distance on electrophoresis as the microsomal metabolite. The hydrolysis product also had the same R_f-values as the microsomal metabolite, as found by co-chromatography in five paper chromatographic systems which differed widely in composition and pH values. The hydrolysis product, extracted from an acidic solution with ether, had a mass spectrum identical to that of authentic dichloroacetic acid. Characteristic mass spectral peaks of m/e 128 (M^+, 2 Cl) (very weak), 84 (M^+–CO_2, 2 Cl), 49 (m/e 84–Cl, 1 Cl) and 48 (m/e 84–HCl, 1 Cl) were observed. The microsomal metabolite was isolated by ether extraction of an acidified, scaled-up reaction mixture and purified by column chromatography on a column (2·5 × 100 cm) of DEAE–Sephadex A-50 with a gradient of 0·02–0·4 M NH_4HCO_3.[4] This product gave a mass spectrum similar to that for the hydrolysis product and for authentic dichloroacetic acid, with major peaks of m/e 84 (2 Cl), 49 (1 Cl) and 48 (1 Cl); interfering impurities prevented unambiguous identification of the molecular ion (M^+ 128, 2 Cl).

An amidase cleaving leucyl-β-naphthylamide is present in the isolated plasma membrane of mouse liver and hepatoma.[5,6] The deacetylation of acetanilide and phenacetin is catalyzed by an amidase purified from hog liver microsomes,[7,8] and the monoethyl analogue of lidocaine is cleaved by an amidase in rabbit liver.[9] The enzyme responsible for liberating dichloroacetate from chloramphenicol may be the same as that hydrolyzing acetanilide, phenacetin and lidocaine.

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Effect of chronic exposure to carbon disulphide upon some components of the electron transport system in rat liver microsomes

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A SINGLE oral dose[1] or short inhalation[2,3] of carbon disulphide leads to the inhibition of oxidative metabolism of foreign compounds in rat liver microsomes. Bond and De Matteis[1] proved that these changes are accompanied by the decrease of the microsomal level of cytochrome P-450. This, however, was not confirmed by the recent data reported by Freundt and Schauenburg.[4] The present studies were concentrated on the electron transport system in rat liver microsomes in the early stage of the chronic CS_2 intoxication.

The studies were carried out in two separate laboratories within 10-month intervals. The first experiment had been performed in January and February 1971 in the Institute of Industrial Hygiene and Occupational Diseases, Prague (Experiment 1) while the second one was carried out in November and December of the same year in the Institute of Occupational Medicine in Łódź (Experiment 2). In the first experiment male albino rats (SPF) were exposed in a toxicological chamber to CS_2 vapours at a concentration of 1·2 mg/l of air for 6 hr daily, 5 days a week. In the second experiment male albino rats (Wistar) were exposed to CS_2 at a concentration of approximately 1·5 mg/l of air for 5 hr daily, 6 days a week. The control animals were kept over the same time period without exposure. In both experiments rats were sacrificed in the 6th and 7th week of the experiment, immediately after termination of the daily exposure. After decapitation, the liver was immediately removed and washed with cold 1·15% KCl, blotted and weighed. Liver microsomes were isolated from homogenate prepared in cold 1·15% (w/v) KCl in the way described by Bond and De Matteis.[1] Following the last centrifuging at 78,000 *g*, the microsomal pellet was suspended in 0·02 M phosphate buffer pH 7·5.

The amount of cytochrome b_5 was determined from the difference spectra of the microsomal suspensions (2·5 mg protein/ml) in NADH reduced and oxidized state. An extinction coefficient of 185 mM^{-1} cm^{-1} for the difference in absorbance at 424 and 409 nm[5] was used. The amount of cytochrome P-450 was determined from the difference spectra between the microsomal suspension (1·25 mg protein/ml) reduced with $Na_2S_2O_4$ and subsequently treated with carbon monoxide and the $Na_2S_2O_4$-reduced control: an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbancy at 450 and 490 nm[6] was used. All spectra were recorded with a Unicam SP 800 spectrophotometer with SP 850 Scale Expansion Accessory and slave recorder EZ 3 (manufacturer: Laboratorní Přístroje, National Enterprise, Praha).

The NADPH cytochrome *c* and NADPH ferricyanide reductase activity was measured according to the method of Williams and Kamin,[7] and the NADH cytochrome *c* and NADH ferricyanide reductase activity was measured according to the method of Jones and Wakil.[8] An extinction coefficient of 1·02 mM^{-1} cm^{-1} at 420 nm for ferricyanide[9] and of 21·1 mM^{-1} cm^{-1} for the difference in the reduced and the oxidized cytochrome *c* at 550 nm[10] were used in calculations.

Proteins were determined by the method of Lowry *et al.*[11] using bovine serum albumin, fraction V as standard.

Five to seven weeks exposure of rats to carbon disulphide at a concentration of 1·2–1·5 mg/l did not result in any symptoms of chronic poisoning. The body weight of the exposed animals was also unchanged as compared with that of controls. However, the exposure resulted in an increase of the liver weight by approximately 14 per cent as compared with the control (Table 1). This phenomenon