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Changes in Kinetic Characteristics of Sheep Liver Microsomes Following Induction and Dietary Stress[1]

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Changes in Kinetic Characteristics of Sheep Liver Microsomes Following Induction and Dietary Stress. WILSON, D. W. AND HANSEN, L. G. (1975). *Toxicol. Appl. Pharmacol.*, 31, 114–119. The effects of dietary stress and polychlorinated biphenyls (PCB) on the kinetic characteristics of ovine liver microsomal oxidases were studied. Curvilinear Lineweaver–Burke kinetic plots were obtained for the *O*-deethylation of *p*-nitrophenetole. Increasing linearity of the plots resulted from microsomal induction due to both dietary stress and PCB exposure. This suggests two enzyme systems with different magnitudes of induction catalyzing the same reaction.

Much current work concerning the hepatic microsomal mixed function oxidase system involves the possibility that there may be more than one terminal oxidase or reactive site; evidence has been gained from comparative metabolic studies (Lin *et al.*, 1973), inhibition kinetics (Sasame and Gillette, 1970, Hansen and Hodgson, 1971) cytochrome P-450 binding kinetics (Hewick and Fouts, 1970), and the discovery that kinetic parameters (K_m and V_{max}) vary with the range of substrate concentration (Hayes *et al.*, 1973).

Previously observed curvilinear Lineweaver–Burke plots could be expected from multiple-enzyme systems catalyzing the same reaction (Wilkinson, 1968; Hansen *et al.*, 1974). A variety of stresses could selectively alter specific systems, changing the degree of curvature of the Lineweaver–Burke plot. We have used the reported effects of chemical induction by polychlorinated biphenyls (Chen and DuBois, 1973) and induction by dietary stress (Paine and McLean, 1973) to attempt to selectively affect individual components of the system.

METHODS

Feeder lambs between 3 and 5 months of age were purchased from commercial sources, individually housed in 4-ft-sq pens and fed the specified diet for 15 weeks. Diets were formulated to be high in fat (6%), low in protein (10.75%), and normal

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(3% fat, 14.5% protein) compared to ideal feed lot rations. Each dietary regime was further subdivided into three groups: control, 20 ppm Aroclor 1242, and 20 ppm Aroclor 1254. After being fed the prescribed diet for 15 weeks, animals were sacrificed by electrical stun and exsanguination. Liver microsomes were prepared by differential centrifugation in 0.05 M Tris buffer (pH 7.4) containing 0.15 M KCl as previously described (Hansen *et al.*, 1974).

If a concentrated microsomal suspension is stored under nitrogen in sucrose solution at −20°C, CO spectral binding characteristics are retained for several weeks (R. M. Philpot, NIEHS, Research Triangle Park, NC, personal communication). Preparations were stored by resuspending the microsomal pellet to 1 g fresh weight/ml in 0.25 M sucrose, gassing with nitrogen for 30 sec, and flash freezing in a dry ice–acetone mixture. This allows storage of microsomes for several weeks with little loss of *O*-dealkylation activity.

For assay, microsomal preparations were diluted to 100 mg fresh weight/ml with Tris buffer (pH 7.4). Incubations were carried out in 20-ml vials at 39°C in a Dubnoff metabolic shaker. Substrate (*p*-nitrophenetole recrystalized from ethanol) was added in 0.05 ml of methyl cellosolve. The reaction was initiated by adding 1 ml of diluted microsomal preparation to 3 ml of Tris buffer–cofactor solution. Final concentrations in 4 ml were as follows: microsomal protein, 1.0–1.8 mg; Tris, 0.05 M, pH 7.4; glucose-6-phosphate, 2.5 mM; NADP, 0.5 mM; glucose-6-phosphate dehydrogenase, 1 unit; $MgCl_2$, 7.5 mM. Reactions were terminated after 15 min by addition of 1 ml of 1.0 N HCl. The product, *p*-nitrophenol, was extracted with 5 ml chloroform and 500 mg NaCl. The product was then extracted from 4 ml of the chloroform extract into 3 ml of 0.1 N NaOH and the highly colored ion quantitated spectrophotometrically at 400 nm with a Bausch and Lomb Spectronic 70. Protein was determined by a modification of the method of Lowry *et al.* (1951), in which sodium citrate is substituted for sodium tartrate.

Kinetic data were plotted according to the method of Lineweaver–Burke double-reciprocal plots (1/specific activity vs 1/substrate concentration). Tangents to each tail of the curve were fitted by least-squares regression analysis.

RESULTS

Increased microsomal *O*-decthylation activity was observed as a result of both dietary and polychlorinated biphenyl (PCB) influences. Specific activity for dietary effects ranged as follows: high fat > low protein > normal. The higher chlorinated PCB, Aroclor 1254 (54% chlorine), resulted in higher specific activity than the lower chlorinated PCB, Aroclor 1242 (42% chlorine). This agrees with data published for PCB induction of *o*-demethylation in rats (Chen and DuBois, 1973).

Both PCBs significantly increased activity over control in the normal and low protein diets (Table 1). Animals on the high-fat diets showed variable responses to PCB, caused in part by the additional stress of urinary calculi. There was a 20% observed incidence of urolithiasis in the high-fat diets. Since high-fat diets predispose sheep to urolithiasis, the stress of this condition could account for the discrepancies observed in this group. In all cases, greater induction was observed at low rather than high substrate concentrations.

Lineweaver–Burke plots which were sharply curvilinear were obtained for control

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TABLE 1

SPECIFIC ACTIVITY OF *p*-NITROPHENETOLE *O*-DEETHYLATION COMPARED TO THE SLOPE OF LINEWEAVER-BURKE KINETIC PLOTS AT LOW (0.0125–0.15 mM) AND HIGH (0.15–2 mM) SUBSTRATE CONCENTRATIONS

Treatment	Specific activity[a]			Slope	
	0.05 mM[b]	0.5 mM	1 mM	0.15–2 mM	0.0125–0.15 mM
Normal control	2.67	6.98	7.48	0.0233	0.0035
Normal 1242	4.07	6.66	7.61	0.0103	0.0022
Normal 1254	5.67	9.48	11.22	0.0074	0.0024
Low-protein control	5.44	8.40	10.36	0.0177	0.0025
Low-protein 1242	9.26	13.07	14.94	0.0094	0.0022
Low-protein 1254	13.72	16.82	18.35	0.0030	0.0023
High-fat control	10.70	16.94	17.31	0.0030	0.0023
High-fat 1242	4.55	7.36	7.30	0.0089	0.0023
High-fat 1254	8.42	12.60	14.08	0.0092	0.0034

[a] nmol/min/mg protein.
[b] Substrate concentration.

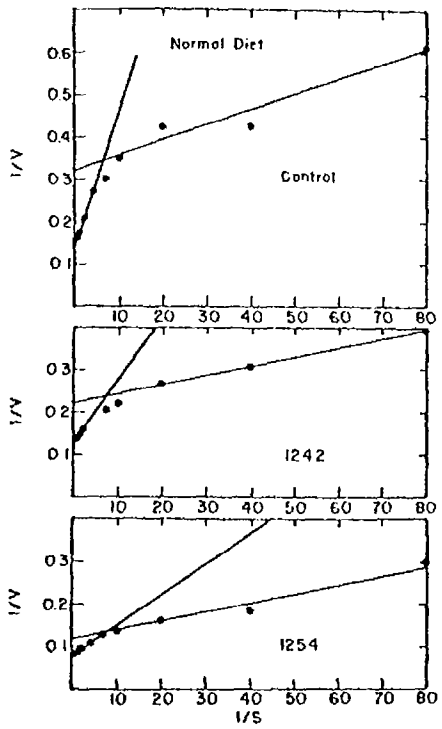

FIG. 1. Effect of 20 ppm Aroclors 1242 and 1254 in normal diet on Lineweaver-Burke double-reciprocal plots. Abscissa 1/*p*-nitrophenetole (mM); ordinate, 1/nmol *p*-nitrophenol produced/min/mg protein.

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animals on normal diets. As the degree of induction due to PCB increased, Lineweaver–Burke plots began to approach linearity (Fig. 1). Similarly, induction resulting from dietary effects resulted in plots closer to linearity. In general, the amount of induction observed in each group correlates with the approach of the Lineweaver–Burke plots to linearity (Figs. 1–3). These results are summarized in Table 1, comparing the specific activity to the slopes of the tangents to each tail of the curve.

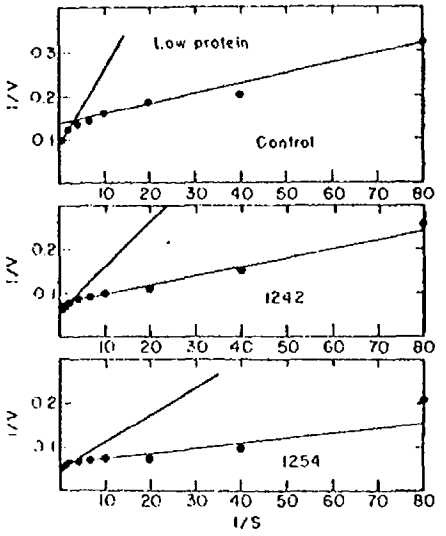

FIG. 2. Effect of 20 ppm Aroclors 1242 and 1254 in low-protein diet on Lineweaver–Burke double-reciprocal plots. Abscissa, 1/*p*-nitrophenetole (mM); ordinate, 1/nmol *p*-nitrophenol produced/min/mg protein.

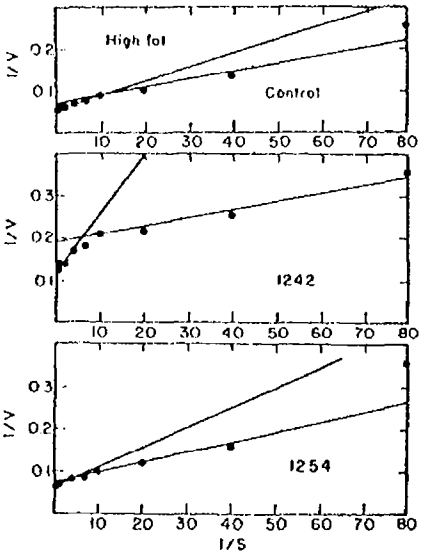

FIG. 3. Effect of 20 ppm Aroclors 1242 and 1254 in high-fat diet on lineweaver–Burke double-reciprocal plots. Abscissa, 1/*p*-nitrophenetole (mM); ordinate, 1/nmol *p*-nitrophenetole produced/min/mg protein.

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DISCUSSION

There are several possible explanations for curvilinear Lineweaver-Burke plots in this system. The first is the possibility of two separate P-450 enzyme systems with different affinities for substrate. The changes due to induction which we observed would support this. Selective induction of one enzyme system would increase its contribution and reduce the effect of the second, noninduced system on the kinetic plot. In our data, the apparently noninduced enzyme system affects the kinetic curve at high substrate concentrations and is therefore a low-affinity enzyme, while the induced system could have a high substrate affinity.

Some authors observing curvilinear kinetic plots using binding kinetics have been unable to eliminate the possibility of a diheme P-450 (Hewick and Fouts, 1970) as an explanation of this phenomenon. Such a system would be inconsistent with the results presented here. Induction of one enzyme with two sites would not result in a change in the shape of the kinetic curve such as we observed.

Many authors have observed curvilinear plots with several different substrates (Lin *et al.*, 1973; Sesame and Gillette, 1970; Hansen *et al.*, 1974; Hayes *et al.*, 1973). P-450 binding studies have also resulted in curvilinear plots (Hewick and Fouts, 1970). The possibility of a separate enzyme system from the NADPH-dependent mixed function oxidases, however, cannot be discounted.

An additional alternative hypothesis which would explain the effects observed here is the possibility of allosteric effects of some exogenous compound or the substrate itself on a single-enzyme system. If a high substrate-to-enzyme ratio increased the rate of metabolism by an allosteric effect, curvilinear kinetic plots would result. Induction of the enzyme system would decrease this ratio, resulting in less deviation from linearity of the kinetic plot.

Many authors are interested in the possibility of multiple enzyme sites in the microsomal mixed function oxidase system. We feel that these data suggest that the system behaves kinetically like a dual-enzyme system.

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