Title

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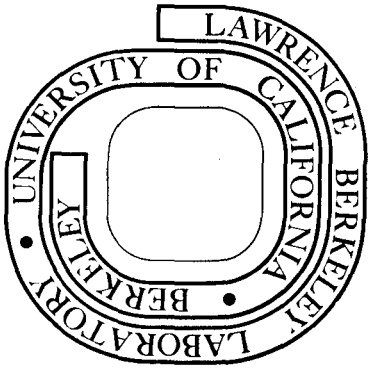

DISCLAIMER

FATTY ACID REQUIREMENTS AND TEMPERATURE DEPENDENCE OF MONOOXYGENASE ACTIVITY IN RAT LIVER MICROSOMES

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SUMMARY

The effect of variation in the microsomal membrane fatty acid composition on Arrhenius plot phase transition temperatures for p-nitroanisole O-demethylation and benzo(a)pyrene hydroxylation has been investigated. In liver microsomes from normal-dieted rats, the cytochrome P450-dependent enzyme activity has a break temperature at 24°C, while that of the P448-dependent enzyme occurs at 29°C indicating that these two enzymes may exist in different patches of membrane. In microsomes having diet-altered fatty acid compositions, benzo(a)pyrene hydroxylase has a break temperature at 33°C, a value higher than that observed in normal-dieted rats. This observation correlates with the increase in saturation observed in the diet-altered fatty acid composition and thus may correspond to a phase transition roughly dependent on the fatty acid melting point. Phenobarbital-induced levels of p-nitroanisole O-demethylase in normal microsomes were 6 times higher than those in microsomes having diet-altered composition, whereas 3-methylcholanthrene-induced levels of benzo(a)pyrene hydroxylase were similar regardless of diet. The low level of P450-dependent enzyme activity in membranes with altered fatty acid compositions suggests that a particular type(s) of fatty acid was not present in sufficient quantity to permit the induction of maximal enzyme activity. Since the induced P448-dependent enzyme activity was the same regardless of diet, there was presumably sufficient quantities of the appropriate types of fatty

acids present in the membrane for induction of this type of activity. Therefore, particular types of fatty acids may be necessary for the induction of maximal activity of particular enzymes in the mixed function monooxygenase system. Our data together with reports in the literature indicate that linoleic acid incorporated in phosphatidylcholine may be required for P450-dependent enzyme activity, while a more saturated acid may serve the same role for P448-dependent enzyme activity.

INTRODUCTION

The mixed function monooxygenase system metabolizes drugs and carcinogens in mammalian tissue[1,2]. Several investigations have identified lipid, cytochrome P450, and NADPH cytochrome P450 reductase as the three components required to obtain maximal monooxygenase activity in a solubilized reconstituted system[3-7]. The lipid has been further identified as phosphatidylcholine, with the type of diacyl chains influencing the amount of reconstituted enzyme activity[3]. Furthermore, dietary lipid has been shown to have an effect on rat liver microsomal mixed-function monooxygenase activities[8,9]; rats fed a fat-free diet had less cytochrome P450 content and lower levels of drug-metabolizing enzymes than those fed a normal diet.

Studies of the kinetics of chemical modification of the reductase in microsomes[10], as well as temperature dependent measurements of microsomal[11-13] and liposomal[14] membrane bound enzyme activities showing break temperatures have supported the proposal that lateral mobility of the reductase and the cytochrome proteins in the lipid is the mechanism whereby several cytochromes can be reduced by one reductase.

Using physical techniques such as spin-label and fluorescence polarization measurements, a succession of transition temperatures has been measured in the endoplasmic reticulum membranes from rat liver[15] and from cells grown in culture[16]. In addition, Arrhenius plots of rat liver microsomal membrane bound enzyme activities have yielded various break temperatures depending on the enzyme assayed[15,11-13].

In this paper we report the effects of diet-altered rat liver microsomal membrane fatty acid composition on changes in p-nitroanisole O-demethylation and benzo(a)pyrene (BaP) hydroxylation. We also measured the Arrhenius plot break temperatures of the enzyme activities in microsomal membranes with different fatty acid compositions. Our results suggest that the lipid fatty acid requirement of each of these enzyme systems is different and that the P450- and P448-dependent enzymes may exist in different patches of microsomal membrane.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley weanling rats (Simonsen Laboratories, Gilroy, CA) weighing approximately 60 g were fed a balanced diet for a few days after arrival. They were then placed in groups, half the group received a normal balanced diet (Berkeley diet containing 7% fat from Foodstuffs Processing Co., Laboratory Animal Food Div., San Francisco, CA) throughout while the other half were fasted for 2 days and then refed a fat-free diet (Fat-free Diet #11270T from U.S. Biochemical Corp., Cleveland, Ohio) for 5-6 days. Both diets contained supplemental vitamins and minerals. Food and water were accessible ad lib throughout, except for food during the fast.

The groups of normal-dieted, and fasted-and- refed rats were each subsequently divided into groups of 4-6 animals. One group received a daily intraperitoneal injection of sodium phenobarbital (PB) (80 mg PB/kg body weight) in 0.9% saline for 3 days prior to

sacrifice while another group received a single intraperitoneal injection of 3-methylcholanthrene (MC) (25 mg MC/kg body weight) in 0.5% methylcellulose in saline one day prior to sacrifice. Control groups received saline, methylcellulose, or nothing. The animals were sacrificed by decapitation and the livers were immediately removed and chilled in ice-cold saline. Microsomes were prepared in batches of 4-6 livers as previously described[17], except the final microsomal pellet was washed once.

Analytical Procedures. The temperature dependence of enzyme activity was obtained by incubating the reaction mixtures simultaneously at various temperatures between approximatley 5°C and 50°C. This was accomplished by performing the incubation in water filled holes which were drilled into an aluminum bar approximately 1.5 m long with a cross section of 7.5 cm x 10 cm. A heat source at one end and a heat sink at the other provided the necessary temperature gradient along the bar. Incubation temperatures were monitored along the bar in control reaction mixtures with a copper-constantan thermocouple and mercury thermometers. A least squares fit of the data to two straight lines was done on a Sigma 2 computer.

BaP Hydroxylase Assay. This assay was basically that of Nebert and Gelboin[18,19] and is described in detail elsewhere[20] except that the substrate was added in 10 µl DMSO to give a concentration of 4 µM. Following a 4-minute pre-incubation period for temperature equilibration, the reaction was initiated

by the addition of 50 µg microsomal protein in 100 µl of Tris buffer pH 7.5. The samples were incubated for 10 minutes at all temperatures and under these conditions the reaction was linear with respect to time and protein. The reaction was stopped by the addition of 1.0 ml acetone. Controls had acetone added at the start of the reaction. The effect of temperature on pH was found to be negligible.

p-nitroanisole O-demethylase assay. This assay is based on the procedure reported by Netter and Seidel[21]. The reaction mixture consisted of 3 mM glucose-6-phosphate, k0 units glucose-6-phosphate dehydrogenase, 1 mM p-nitroanisole, 1 mg protein, and 1 ml of 25 mM phosphate buffer pH 7.85. A 1.0 ml volume was pre-incubated for 3 minutes at the specific temperature. The reaction was started by addition of 0.025 mole $NADP^+$ in 10 µl buffer and it was stopped by addition of 200 µl of 1N NaOH. The controls had the NaOH added at the start of the reaction.

The NaOH also served to clarify the assays presumably by dissolving the protein and thus reduced one cause of the light scattering. The absorbance of the reaction product, p-nitrophenol, was measured at 400 nm using the control as a reference in a Cary 118 spectrophotometer. The absorbance of the substrate, p-nitroanisole, at 400 nm was negligible. An effective molar extinction coefficient of 1.5×10^4 $(M\ cm)^{-1}$ was determined by measuring the change in absorbance at 400 nm upon addition of known amounts of

p-nitrophenol after the reaction was stopped. The reaction was generally run for 3-5 minutes and was linear at all temperatures used. The non-linear region usually seen at the start of the O-demethylation of p- or o-nitroanisole according to Netter[22] is not observed under the conditions described above. The reaction was linear in protein up to 1 mg at all temperatures used in this study. Addition of nicotinamide to obtain maximum rates was found to be unnecessary. Indeed, when 20 μM nicotinamide was present in the reaction mixture the measured velocities were approximately 30% lower, presumably due to inhibition by nicotinamide[23].

Fatty acid compositions. Lipids were extracted from liver microsomes equivalent to 10 mg of microsomal protein in chloroform, methanol, and water (8:4:3, V:V:V) by vortexing for several minutes[24]. The aqueous layer was removed by suction and the chloroform layer evaporated to dryness under nitrogen and the residue dissolved in 2 ml chloroform for loading onto a silicic acid column. The column was 5 mm inside diameter and contained approximately 6 grams of activated silicic acid 100 mesh (Mallinckrodt, Inc., St. Louis, MO). The column was washed with 30 ml chloroform before each sample was run. The sample was then eluted with 10 ml chloroform (neutral lipid, fraction 1) and 10 ml methanol (complex lipid, fraction 2). The flow rate was approximately 0.5 ml/min. Both fractions were evaporated to dryness and then saponified by heating to 100°C in a capped tube for 1 hour in

2 ml of 2N KOH in ethanol-water (1:1). Free cholesterol was extracted from fraction 1 with 3 x 10 ml petroleum ether. The combined ether extracts were evaporated to dryness with nitrogen and the cholesterol when silylated was heated in a capped tube to 65°C for 10 minutes in the presence of 100 µl pyridine, 300 µl N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), and 25 µl trimethylchlorosilane (TMCS). The reaction mixture was evaporated to dryness with nitrogen and immediately dissolved in 0.5 ml chloroform for gas chromatographic analysis. Some samples were run without silylation. The aqueous phase of fraction 1 was acidified with H_2SO_4 and the fatty acids extracted with 10 ml petroleum ether. The organic phase was washed three times with water and evaporated to dryness with nitrogen prior to methylation.

The non-saponifiable lipid was extracted from fraction 2 with 10 ml petroleum ether. The saponified fatty acids were then acidified, extracted, washed, and dried as described above for fraction 1. The fatty acids from fractions 1 and 2 were then separately methylated by heating to 100°C for 4 minutes in a capped tube in the presence of 2 ml benzene and 2.5 ml BF_3 (14% in methanol). The reaction was stopped by addition of 10 ml water. The methyl esters were extracted with 10 ml heptane, evaporated to dryness with nitrogen and dissolved in 200 µl ethyl acetate for gas chromatographic analysis.

Gas-chromatographic (GC) analysis. The analyses of the methyl esters of fatty acids were performed on a Varian Model 2700 gas chromatograph at 180°C, with helium carrier gas flowing

at 6 ml/min. A 10-foot long, 0.030-inch inside diameter glass capillary column containing HI-EFF-2BP (Applied Science Labs) on 100/120 mesh Gaschrom Q was used. Peaks were identified by comparison with mobilities of known standards and by computerized gas chromatographic-mass spectrometric (C-GC-MS) analysis. The C-GC-MS analyses were performed using a Dupont 491-2 double focusing mass spectrometer coupled directly to a Varian aerograph 204 GC equipped with the same glass capillary column described above. Cholesterol levels in the microsomal membranes were measured by extraction and GC analysis of its silyl-derivative. A Hewlett-Packard GC model 5750 was used with temperature programmed from 130°C to 300°C at 30°C/min. Carrier gas was helium flowing at 12 ml/min. A 12-foot long, 1/16 inch inside diameter stainless steel column containing 3% OV-17 (Applied Science Labs) on Chromosorb W 100/120 mesh was used. Known amounts of silylated cholesterol were used as standards.

Cytochrome P450 and P448 content was measured by the method of Omura and Sato[25] using a molar extinction coefficient of 91$(mM\ cm)^{-1}$ and protein was measured by the method of Lowry[26] using bovine serum albumin as a standard.

All solvents used were chromatoquality (MC/B, Norwood, OH). GC standards were purchased from Sigma Chemical Co. (St. Louis, MO) and checked for purity initially by thin layer chromatography [27].

RESULTS

Lipid analysis. The relative (mole percent) composition of the major saponified fatty acids from rat liver microsomes maintained on

a normal diet and those fasted for 48 hours and then refed a fat-free diet is shown in Table I. The fatty acid composition is shown for non-induced animals, as well as for those induced with phenobarbital and with 3-methylcholanthrene. The data in Table I indicate that the inducing compounds do not have a measurable effect on the fatty acid composition of rat liver microsomes. Microsomes from the rats maintained on the two different diets, however, have markedly different fatty acid compositions. Compared to the normally fed animals, the fasted and refed animals have elevated levels of palmitoleic acid (16:1), oleic acid (18:1), and eicosatrienoic acid (20:3), and decreased levels of the polyunsaturated acids 18:2 and 20:4, which is in agreement with results reported by others[28,29]. There is an overall increase in saturation of the fatty acids in the animals that were fasted and refed the fat-free diet. These diet-induced changes in fatty acid composition are similar to those observed in rats fed a diet deficient in essential fatty acids[30].

When the fraction 1 free fatty acids were analyzed (data not shown), changes in relative composition were observed similar to those measured in the fraction 2 saponified fatty acids. Total cholesterol levels were also measured, and rats fed the normal diet had approximately twice that of the rats fasted and refed the fat-free diet. (Normal diet: 0.030 μmol cholesterol/mg protein; fasted and refed: 0.014 mol cholesterol/mg protein.)

Temperature dependence. In normal-dieted rats induced with PB the break point in the Arrhenius plot of p-nitroanisole O-demethylation (Fig. 1) is at 24.2 ± 2.1°C (n = 5, 95% confidence)

which agrees with the break temperature of 23.9 ± 1.6°C recently reported by Yang and coworkers[13]. In contrast, the P448-dependent BaP hydroxylase (Fig. 2) has a significantly different break temperature at 29.3 ± 1.2°C (N = 3, 95% confidence) in normal-dieted MC-induced and non-induced rats suggesting that these two enzymes may be located in different environments within the membrane.

The temperature dependence of BaP hydroxylase activity was also measured in microsomes from animals which were fasted and refed the fat-free diet (Fig. 3). These microsomal membranes have an overall increase in saturation in their fatty acids, and have a significantly (99% confidence) higher break temperature at 33.5 ± 0.1°C (n = 2, 95% confidence). Owing to low levels of activity, the temperature dependence of p-nitroanisole O-demethylase could not be measured in PB-induced microsomes from rats fed the fat-free diet nor in non-induced microsomes regardless of diet.

In Figs. 1-3, the calculated activation energies above and below the break temperature are shown. In all cases regardless of enzyme type or membrane fatty acid composition, the value of the activation energy above the break temperature is less than that below it. Electron transfer, the first or the second electron, has been considered as the rate limiting step in the overall enzyme catalyzed reaction[10-13,31-35]. Since the membrane is in a more fluid state above the phase transition temperature this may facilitate electron transfer between the reductase and the cytochrome, resulting in a smaller activation energy above the . transition. Presumably electron transfer rates would vary between

different patches of lipid (or fatty acid) and thus might explain the large variation in metabolic rates from approximately 0.3 nmol $(min.mg)^{-1}$ for BaP hydroxylation to approximately 30 $nmol(min.mg)^{-1}$ for benzphetamine N-demethylation[13].

Induced enzyme activity. In rats fed a normal diet the levels of MC-induced BaP hydroxylase activity are approximately the same as those in membranes with altered fatty acid compositions. Altered membrane fatty acid composition apparently does not affect the ability of the animal to synthesize the BaP hydroxylation enzyme in the monooxygenase system. However, PB-induced levels of p-nitroanisole O-demethylation activity in normal-dieted rats are approximately 6 times greater than in rats with diet-altered fatty acid compositions, thus suggesting that membrane fatty acid composition may affect the ability of the rat to synthesize certain types of enzymes in the mixed-function monooxygenase system.

Cytochrome P450 and P448 content was measured by absorption of the reduced protein-CO complex and this data is shown in Table II. Since multiple forms of the cytochrome have been shown to exist[36,39], it is difficult to interpret. For example, in rats refed the fat-free diet, the specific activity of p-nitroanisole O-demethylation increases upon PB-induction while the measured cytochrome content remains essentially constant. It is possible that the cytochrome content consists of different forms of the cytochrome before and after induction.

DISCUSSION

The results reported herein suggest that the physical characteristics of the endoplasmic reticulum membrane affect the activity of those enzymes bound to it. This type of behavior has also been observed in purified and reconstituted enzyme systems. Strobel and coworkers[3] have shown that P450-dependent benzphetamine hydroxylation in a reconstituted system requires phosphotidylcholine, and the type of fatty acid chains on the phospholipid affected the amount of enzyme activity, while Lu and coworkers[4] came to the same conclusion for P448-dependent BaP hydroxylation.

With normal-dieted rats, the different break temperatures (fig. 1 and 2) for the two enzyme activities suggests that they are associated with different fatty acids and therefore may be physically located in different patches or areas of the membrane. The higher phase transition temperature observed for BaP hydroxylase a activity implies that its fatty acid environment is more saturated than that associated with the p-nitroanisole O-demethylation enzyme. In both control and MC induced fasted and refed rats the break temperature for BaP hydroxylation (Fig. 3) is approximately 4°C higher than that observed in normal-dieted rats. This change in phase transition temperature suggests that the P448-dependent enzyme is in a lipid environment that is sensitive to the overall fatty acid composition of the membrane. A change to more saturated fatty acids is accompanied by an increase in their melting point temperature. This type of compositional change was observed when rats were fasted and refed the fat-free diet, and there was a corresponding

increase in the Arrhenius break temperature for BaP hydroxylase. It is tempting to postulate from this correlation that the fatty acid composition at or near the hydroxylase is influencing the observed changes in phase transition temperature. The role of fatty acids in those enzyme systems is unknown but they may be needed for proper orientation between reductase and cytochrome.

A comparison of the levels of induced enzyme activities (Table II) in microsomes having normal fatty acid composition with those membranes having altered fatty acid composition gives an indication of the fatty acid required for maximal enzyme activity. The fact that the P450-dependent enzyme activity did not increase upon PB induction in the refed animals to the level that was attained in normal-dieted animals suggests that a particular type(s) of fatty acid was not present in sufficient quantity to permit the induction of maximal enzyme activity. On the other hand, the P448-dependent enzyme activity was inducible to a high level in the refed rats. Presumably sufficient quantities of the appropriate types of fatty acids were present in the membrane for induction of this type of activity. Minimum levels of particular types of fatty acids may, therefore, be necessary for induction of maximal activity of particular enzymes in the mixed-function monooxygenase system. It should be noted that the normal diet and the fat-free diet were different and although both contained vitamin and mineral supplements, the possibility exists that the low levels of induced P450-dependent enzyme

activity might be due to some non-lipid dietary factor. Since the induced P448-dependent BaP hydroxylase activity was approximately the same regardless of diet, both diets are sufficient to supply the nutrients necessary for maximal activity of this enzyme system.

In rats fasted and refed the fat-free diet, we observed an approximately 4-fold decrease in total (fraction 2) saponifiable linoleic acid (18:2) compared to normal-dieted rats, with an associated low level of induced P450-dependent p-nitroanisole O-demethylation activity. Davison and Wills[40] found that following PB injection there was an increase in the proportion of linoleic acid in phosphatidylcholine and phosphatidylethanolamine. Our observations, along with theirs, suggests that this fatty acid is necessary for maximal induction of activity of the P450 enzyme system. We did not detect changes in linoleic acid levels upon induction presumably because we were measuring the fatty acids of all complex lipids and not those of phosphatidylcholine and phosphatidylethanolamine.

The increase in linoleic acid observed by Davison and Wills was well correlated with the time courses of increases in cytochrome P450 and reductase content, as well as with aminopyrine demethylase activity. It appears likely, as Davison and Wills point out, that a selected species of phosphatidylcholine containing linoleic acid is essential for the induction of the cytochrome P450-dependent enzyme system.

The situation is somewhat similar for the P448 enzyme system in that Davison and Wills[40] observed an increase in the

proportion of oleic acid in phosphatidylcholine and ethanolamine following MC injection. This increase in oleic acid was well correlated with the time course of increase in biphenyl 4-hydroxylase activity in their experiments, although the correlation of oleic acid increase with cytochrome and reductase increase was not quite exact. In our fasted and refed rats we observed an approximately 4-fold increase in total (fraction 2) saponifiable oleic acid compared to normal-dieted rats, with an associated high level of induced P448-dependent BaP hydroxylase activity in both normal and refed animals. These observations suggest that while linoleic acid is a necessary constituent of the phosphatidylcholine in the cytochrome P450-dependent system, oleic acid or a more saturated fatty acid may be a necessary component of the P448-dependent system.

The model suggested above is further supported by the fact that oleic acid has a melting point several degrees higher than that of linoleic acid and the Arrhenius plot break temperature for the P448-dependent BaP hydroxylase activity occurs at a higher temperature than that of the P450-dependent p-nitroanisole O-demethylase activity. This model implies that the P450- and P448-dependent enzymes may be associated in the membrane with different fatty acid compositions, and may even be in different patches of non-uniform membrane lipid distribution[41], or on different sides of a membrane with an asymmetric lipid distribution[42].

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FIGURE LEGENDS

Figure 1. Arrhenius plot of p-nitronanisole O-demethylation activity in liver microsomes from PB-induced rats fed a normal diet. Specific activity in units of nmol p-nitrophenol $(min.mg)^{-1}$ are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperature are shown in kcal/mole.

Figure 2. Arrhenius plot of BaP hydroxylase activity in liver microsomes from MC-induced (O) and non-induced (Δ) rats fed a normal diet. Specific activity in units of nmol 3-hydroxy-BaP $(min.mg)^{-1}$ are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperatures are shown in kcal/mole.

Figure 3. Arrhenius plot of BaP hydroxylase activity in liver microsomes from MC-induced (O) and non-induced (Δ) rats fasted and refed a fat-free diet. Specific activity in units of nmol 3-hydroxy-BaP $(min.mg)^{-1}$ are plotted on an arbitrarily normalized log activity scale. Activation energies above and below the break temperatures are shown in kcal/mole.

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Table I: Fraction 2 Saponifiable Fatty Acids (Relative Mole Percent)

Diet and Pre-Treatment	16:0	16:1	18:0	18:1	18:2	20:3	20:4	Double Bond
NC	16.2	0.8	31.6	6.3	16.8	0	28.4	1.5
NPB	19.6	1.9	33.5	7.6	16.2	0	20.8	1.3
NMC	17.9	2.0	33.9	6.8	17.7	0	21.8	1.3
RC	24.2	6.7	20.3	29.1	4.8	9.7	7.4	1.0
RPB	19.3	7.1	23.0	28.1	3.4	10.1	9.1	1.0
RMC	18.8	3.8	25.4	28.2	4.3	9.9	9.5	1.1

N = normal diet

R = fasted and refed the fat-free diet

C = non-induced control

PB = phenobarbital-induced

MC = 3-methylcholanthrene-induced

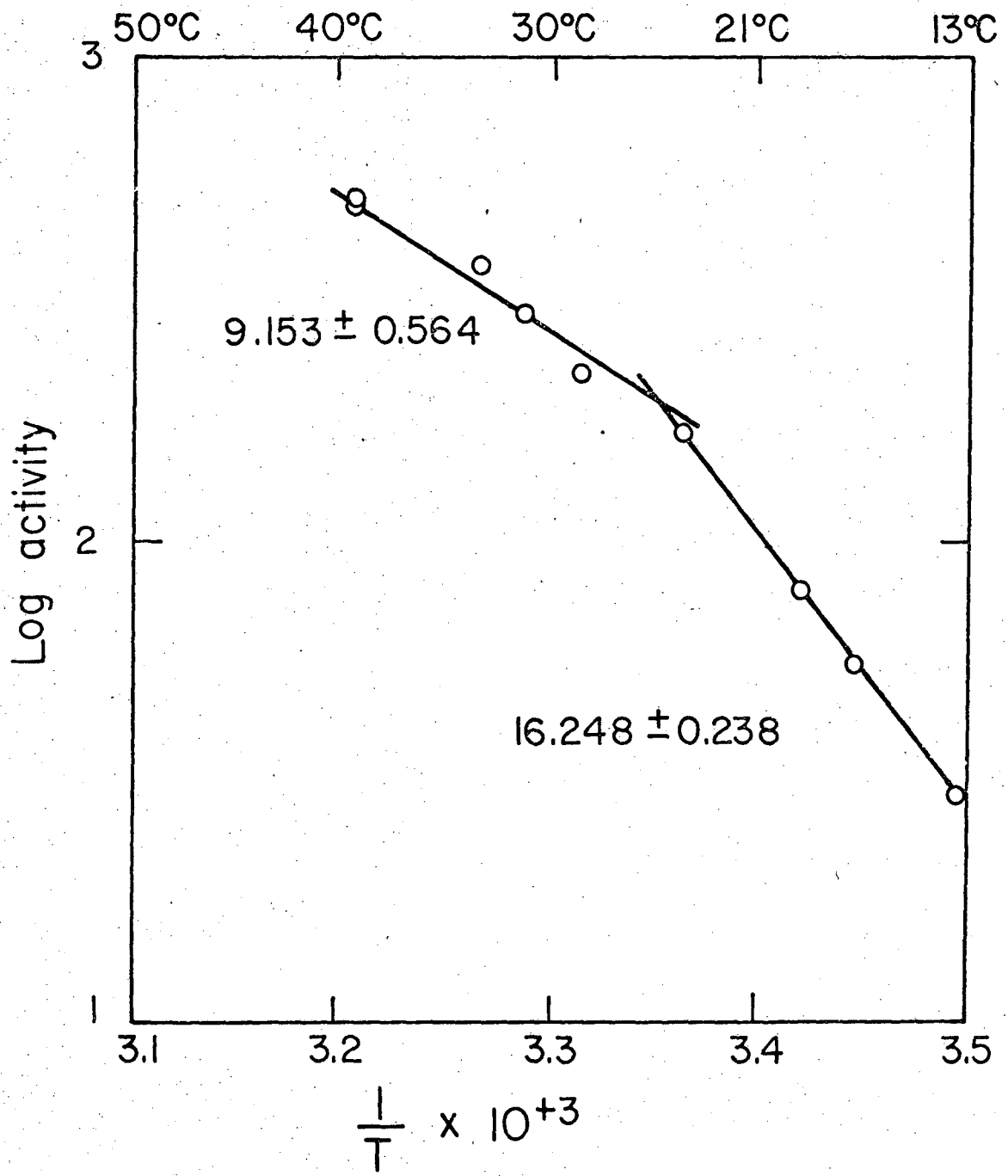
Double bonds = average number of double bonds per fatty acid molecule.

Table II: Cytochrome P450 and P448 Content and Specific Enzyme Activity at 37 C.

Diet and Pre-Treatment(1)	nmol p-nitrophenol / mg min.	CYT P450 Content (nmol/mg Protein)
NC	0.18 ± 0.17	0.38 ± .10
NPB	2.41 ± 0.65	1.41 ± .01
RC	0.02 ± 0.03	0.34 ± .09
RPB	0.42 ± 0.22	0.36 ± .01

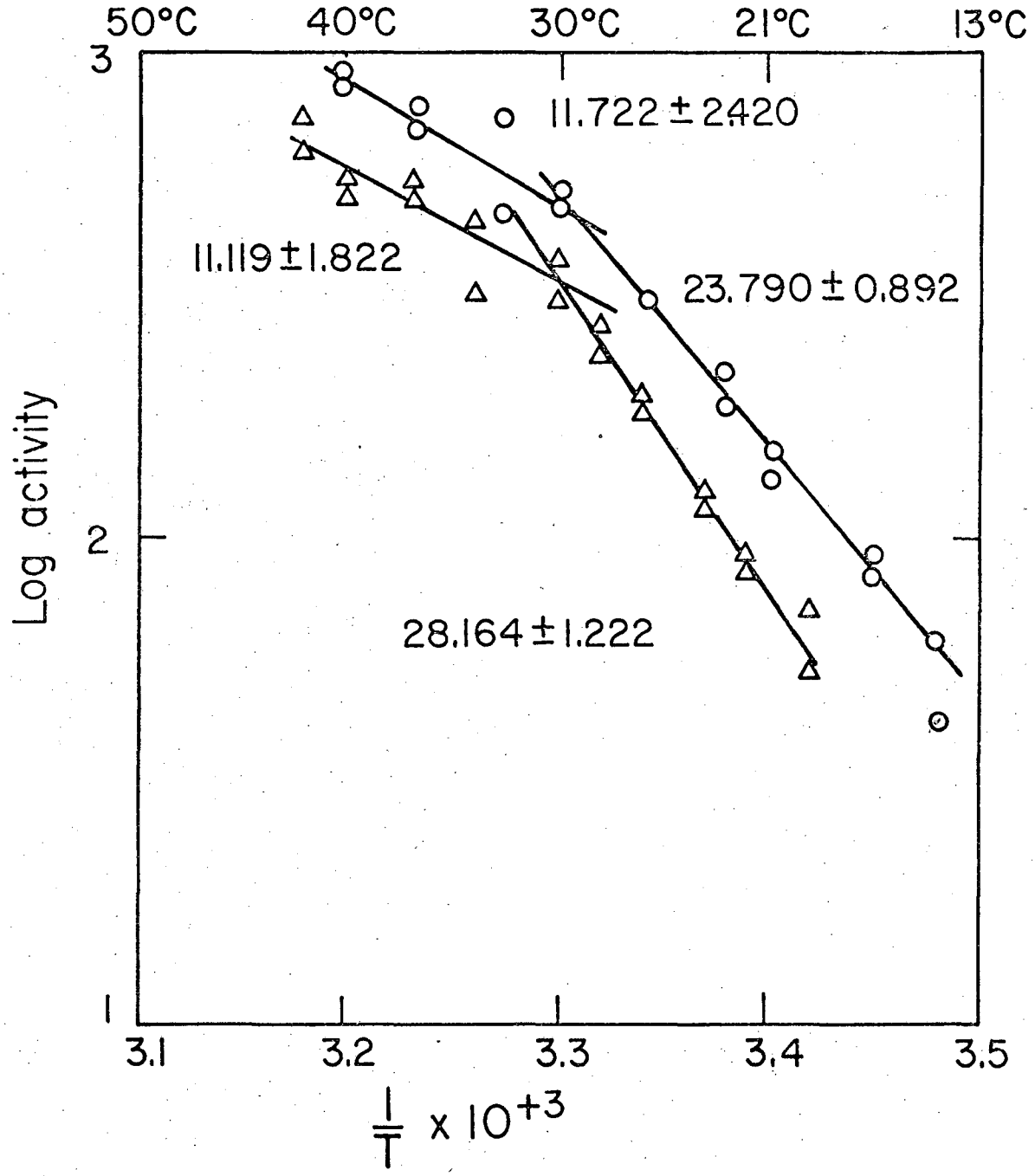
Diet and Pre-Treatment	nmol 3-hydroxy-BaP / mg min	CYT P448 Content (nmol/mg Protein)
NC	0.089 ± .005	0.38 ± .10
NMC	0.225 ± .024	0.98 ± .08
RC	0.033 ± .004	0.34 ± .09
RMC	0.197 ± .018	0.64 ± .04

(1) Nomenclature is the same as in Table I



XBL – 776–4446

Fig. 1 BECKER et al



XBL 776-4448

Fig. 2 Becker et al

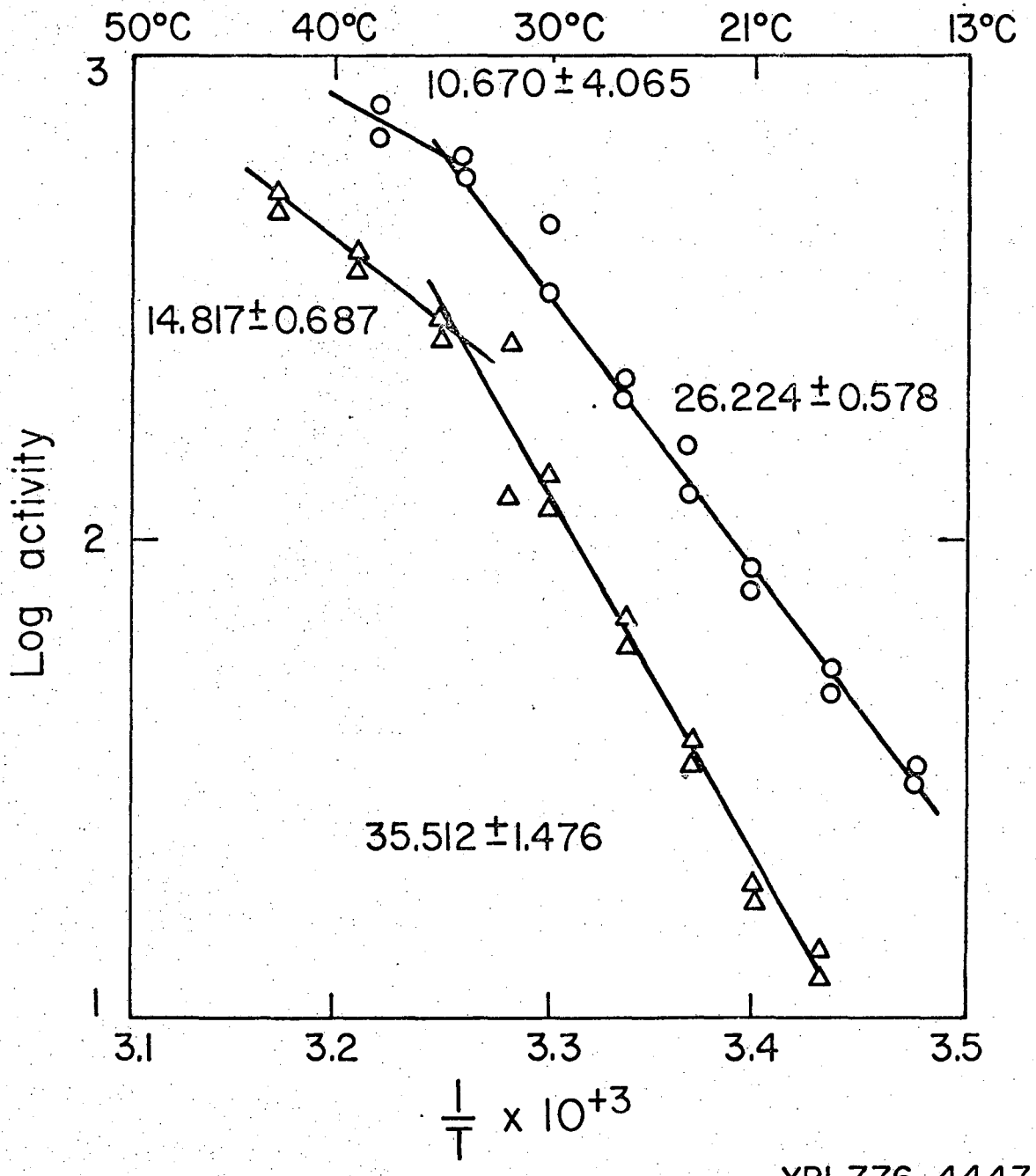


Fig. 3 BECKER et al

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