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The Effect of Metabolites, which Accumulate During Phenylketonuria, on the Activity of Stearoyl-Coenzyme a Desaturase

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THE EFFECT OF METABOLITES, WHICH ACCUMULATE DURING PHENYLKETONURIA, ON THE ACTIVITY OF STEAROYL-COENZYME A DESATURASE

by

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Walter Scott

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INTRODUCTION

The biochemistry of mental deficiency is not well understood. A major approach to illuminating the biochemical basis of retardation has been through the study of phenylketonuria. This line of research has lead to the accumulation of a considerable amount of data concerning phenylketonuria, but the chemical cause of mental deficiency is still much in question.

Phenylketonuria was first described by Følling (2) in 1934. Five years later, Jervis (3) was able to show that phenylketonuria is inherited through a single autosomal recessive gene. The proof of this genetic mechanism was provided by a frequency analysis of the incidence of phenylketonuria within families in which the disease occured. Jervis (4) then demonstrated that phenylalanine accumulated in phenylketonurics. The phenylalanine build-up led to the finding that the metabolic error in this disease lay in the inability to oxidize phenylalanine to tyrosine (5). Jervis (6) also confirmed the above finding when he isolated inactive phenylalanine parahydroxylase from the livers of phenylketonurics.

As a result of the block in the phenylalanine-to-tyrosine metabolic pathway and the concomitant increase in the plasma phenylalanine concentration in phenylketonuria patients, the quantity of phenylalanine that is metabolized by alternative metabolic pathways is increased. The concentration of phenylalanine in the blood of phenylketonurics is thirty times that seen in normals, and such species as phenyl-

acetic acid are detectable in the phenylketonuric's urine (2). Normally, phenylacetic acid is not detectable in the urine (2). Furthermore, since phenylalanine is not converted to tyrosine, the pool of tyrosine available for metabolism is reduced. This results in a reduction of tyrosine derivatives (2), and related metabolic products.

Additionally, the presence of the elevated concentration of phenylalanine found in phenylketonurics inhibits the action of 5-hydroxytryptophan decarboxylase (2). This inhibition manifests itself in a decrease in the level of the decarboxylation product, serotonin. Concomitant with the decrease in serotonin concentration there is an increase in the products from other catabolic pathways of tryptophan. Specifically, there is an increase in the levels of indolepyruvic and indoleacetic acids.

The metabolites, whose levels are either increased or decreased in phenylketonuria, may in some way be related to the major functional abnormality in phenylketonuria: an impaired intellectual ability. Crome, Tymms, and Woolf (7) showed that as the pathology developed and the mental retardation became more severe, there was a correlated demyelination as evidenced by increased myelin breakdown products. These authors also demonstrated a decrease in cholesterol and cerebrosides of white matter. Menkes (9) found a decrease of these lipids in some, but not all, of the phenylketonuric brains he examined. Foote, Allen, and Agranoff (8), however, examined several brain samples from phenylketonurics and did not find a decrease in cholesterol and cerebrosides. These authors found that the ratio of oleic acid to stearic acid was lower in both the white and the

gray matter from phenylketonurics as compared to that in normal individuals.

Biran and Bartley (10) measured the percentage of total fatty acids in rat brains as a function of age. Their data show that there is a pronounced increase in the oleic acid to stearic acid ratio from five day old to adult rats. In the five day old rat brains the oleic acid to stearic acid ratio is less than one, whereas in the adult rat brain this ratio was greater than one. The change in the oleic acid to stearic acid ratio with maturation suggests that a low value of this ratio is correlated with a relatively undeveloped brain. Furthermore, in a study of rats having experimentally induced phenylketonuria, Foote and Tao (11) observed a lowered oleic acid to stearic acid ratio in the brain glycerophosphatides during the period of rapid brain development.

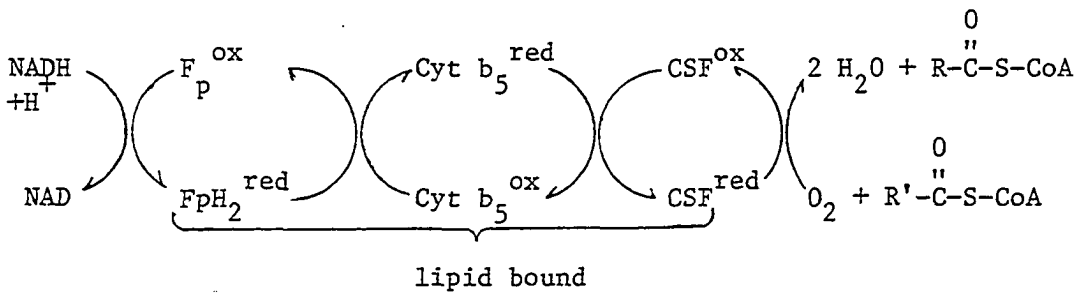
A person's most probable sources of oleic acid are: the desaturation of stearic acid, and the person's diet. The desaturation of stearic acid to oleic acid is catalyzed by a desaturase complex.

This complex is membrane-bound and is isolated in active form together with the microsomal cell fraction. The necessary components for the active complex are: cytochrome b_5 (molecular weight about 16,000 daltons) (12), cytochrome b_5 reductase -- EC 1.6.2.2 (molecular weight about 45,000 daltons) (13), NADH or NADPH (14), molecular oxygen (15), a lipid membrane (16), and stearoyl-CoA desaturase -- EC 1.14.99.5 (molecular weight about 50,000 daltons) (17).

Stearoyl-CoA desaturase is the enzyme involved in the terminal reaction whereby two hydrogen atoms are removed from stearic acid

and a double bond is formed to yield oleic acid. Stearoyl-CoA desaturase contains one atom of non-heme iron as part of its active site (17) and most researchers believe that the active site of stearoyl-CoA desaturase also involves at least one active sulfhydryl group (18). Furthermore, the hydrogen removal from stearic acid in an erythro configuration as is demonstrated by the kinetic isotope studies of Morris (19).

The mechanistic scheme by which the components of the desaturase complex act together to desaturate stearic acid is as follows (20):



where Fp is cytochrome b_5 reductase

Cyt b_5 is cytochrome b_5

CSF (cyanide sensitive factor) is stearoyl (acyl)-CoA desaturase

R' is saturated and usually $C_{17}H_{35}$

R is unsaturated and usually $C_{17}H_{33}$

Thus, given that stearic acid is the precursor for oleic acid, and that the ratio of oleic acid to stearic acid is lowered in phenylketonuria, it seemed plausible that the altered concentrations of metabolites characteristic of the disease may affect the formation of oleic acid from stearic acid. The research reported herein is a survey of the effect of some of these metabolites on the activity of the desaturase complex.

MATERIALS AND METHODS

Chemicals

Carboxyl-labeled {1-^{14}C}-stearic acid and {stearoyl-1-^{14}C}-stearoyl-CoA were purchased from New England Nuclear Corp., Massachusetts. Unlabeled fatty acids, used for diluents and standards, were obtained from Applied Science Laboratories, Inc., Pennsylvania. Metabolites and unlabeled stearoyl-CoA were purchased from Sigma Chemical Co., Missouri. All chemicals, except for the silver nitrate which was of technical grade, were of reagent grade quality and all solvents were redistilled.

Hexane aliquots containing 0.42 μ moles of 1-^{14}C stearic acid were placed in 25 ml culture tubes. The solvent was evaporated and 0.108 μ moles of unlabeled stearic acid in chloroform:methanol (1:1 v/v) were added. The solvent was evaporated and 1 ml of concentrated ammonium hydroxide was added followed by vigorous mixing. The excess reagent was removed with a stream of nitrogen and slight warming. The enzyme assays using ammonium stearate were done in these same tubes.

Isolation of Rat Liver Microsomes

Rat liver microsomes were isolated according to the procedure of Paulsrud, Stewart, Graff, and Holman (21). Rats which had previously been given free access to food and water, were deprived of food (not of H_2O) for 24 hours. Following the food deprivation,

these rats were refed for 21 hours. The food deprivation and the refeeding procedure increases the activity of the stearoyl-CoA desaturase complex isolated from liver microsomes as shown by Oshino and Sato (22).

After refeeding, the rats were killed by cervical dislocation, decapitated and their livers excised. The livers were minced and homogenized in two volumes of 0.25 M sucrose, 0.005 M $MgCl_2$ homogenizing solution at $0^{\circ}C$.

The homogenate was then centrifuged at 15,000 x g at $0^{\circ}C$ for 30 min to remove cell debris, nuclei, and mitochondria. The supernatant was removed with a pipet and then recentrifuged at 50,000 x g for two hours at $0^{\circ}C$. The resulting pellet, containing the microsomes, was resuspended in a volume of homogenizing solution equivalent to the original liver weight. This microsomal suspension was used as the enzyme solution. It was divided into 5 ml aliquots which were frozen in a dry ice-acetone bath and stored at $-20^{\circ}C$ until used.

Protein levels were determined by the Lowry method as described by Chaykin (23). A 0.50 ml aliquot of protein solution was combined with 5.0 ml of a solution containing 0.01% $CuSO_4$, 0.02 % potassium tartrate, 2 % Na_2CO_3, and 0.1 N NaOH, and mixed using a vortex mixer. The protein solution was either 0.02 ml of enzyme solution plus 0.48 ml of water, or 0.50 ml of standard bovine serum albumin solution. After 10 min at room temperature, 0.50 ml of 0.67 N phenol reagent was added. The solution was again mixed using a vortex mixer and kept at room temperature for 10 min. The absorbance was then measured at 600 nm in a Spectronic 20 spectrophotometer.

Enzyme Assays

The stearoyl-CoA desaturase complex was assayed using four different sets of incubation conditions. Three of the assay systems used ammonium stearate and the fourth used stearoyl-CoA as the substrate. Three assays using ammonium stearate were based on the work of Paulsrud et al (21). The assays systems using stearoyl-CoA were based on the work of Holloway (20).

In all cases, the frozen microsomal suspensions were thawed in an ice-water bath and incubations were performed in 25 ml culture tubes in a water bath, shaker at 39°C. This temperature had been reported in the literature to be near the optimum for this enzyme system (21). After incubation, the culture tubes were cooled in an ice-water bath.

Desaturase assays with ammonium stearate as the substrate

In desaturase assay system I the volume of the incubation mixture was 1.5 ml. This volume was 67 mM in pH 7.35 potassium phosphate buffer, 2.5 mM in $MgCl_2$, 125 mM in sucrose, 1 mM in NADH, 50 mM in ATP, 2.5 mM in CoASH, 0.1 mM in ammonium stearate ({1-^{14}C}-ammonium stearate, specific activity 0.32 m Ci/m mole), and contained 3.2 mg microsomal protein. When the assay contained a metabolite, which was tested for its effect on the activity of the desaturase complex, the metabolite concentration was 10 mM. Incubations were for 10 min.

Desaturase assay system II was a modification of system I. In this system the NADH concentration was 2 mM, the ATP concentration 100 mM, and the metabolite concentration, if present was 0.1 mM.

Incubations were for 15 min except for those experiments in which the time course of the reaction was determined.

In desaturase assay system III the concentration of all the components were the same as those in system II, except for metabolites and microsomal protein. No metabolites were present in system III and the concentration of microsomal protein varied from 1.6 mg - 4.0 mg per incubation tube. Incubations were for 15 min.

After the incubation, 1.0 ml of 10 % methanolic HCl was added to each tube of desaturase system I, II, and III assays. To reduce the loss of labeled substrate and product, 2.0 ml of a chloroform: methanol (1:1, v/v) solution, containing 0.25 mg each of stearic and oleic acid, was added. The total lipids were then extracted using the Folch method (24); the addition of 1 ml chloroform and 1 ml water resulted in two layers. The lipids were contained in the lower, organic layer. Centrifugation at 2000 rpm for 10 min left the two layers separated by a protein pellicle. One milliter of the organic layer was removed and dried under a stream of N_2 with a slight warming. A 0.1 ml volume of chloroform:methanol (2:1, v/v) that contained 0.01 % phenolphthalein was used to redissolve the dried lipid extract. The lipids in this solution were transesterified with 1 N sodium methoxide. This was added in sufficient amount to produce a persistent pink color, and then a further excess of about 0.3 volume was added (25). This mixture was stirred, using a vortex mixer and allowed to stand at room temperature for 5 min. After the transesterification, the unesterfied lipids were treated with sufficient 10 % methanolic HCl to remove the pink color, plus a one third volume excess of methanolic HCl (25).

Following vigorous mixing (vortex mixer), the lipids were esterfied at room temperature for one hour. Next, the mixture was again dried under a nitrogen stream with slight warming. This dried mixture was redissolved in 0.10 ml of hexane and spotted on a 5 % silver nitrate TLC plate with a microliter syringe.

The plate was developed twice in the same dimension using a hexane:diethyl ether:acetic acid solvent (94:4:2, v/v/v). The fatty acid methyl esters were visualized by spraying the plates with 0.2 % solution of 2,7-dichlorofluorescein in methanol. The methyl oleate and methyl stearate spots, as identified by comparison with standards, were scraped off the plate and collected in scintillation vials. The vials were filled with 10 ml of scintillation fluid which consisted of toluene containing 4 g of PPO and 0.5 g of POPOP per liter. The radioactivity was determined in a Nuclear Chicago (model Mark II) liquid scintillation counter.

Desaturase assays with stearoyl-CoA as the substrate

All incubations in desaturase assay system IV were performed in a total volume of 1.5 ml. The incubated solution was 67 mM in pH 7.35 potassium phosphate buffer, 2.5 mM in $MgCl_2$, 125 mM in sucrose, 2 mM in NADH, 0.067 mM in stearoyl-CoA ({stearoyl-1-^{14}C}-stearoyl-CoA, specific activity $\frac{2.1 \text{ m Ci}}{\text{m mole}}$), and contained 6.3 mg of microsomal protein. When the assay contained a metabolite, the metabolite was present at one or more of the following concentrations: 0.027, 0.053, 0.080, 0.10, 0.17, 0.33, 0.40, 1.0, 4.0, 10., or 40. mM. Incubations were for 15 min, except when the time course of the reaction was measured.

After the incubation, 1.0 ml of 8.5 % KOH in 95% ethanol was added to each tube. The KOH served to saponify the lipids. The tubes were then capped with glass marbles and placed in a boiling water bath for 20 min. After saponification, the tubes were cooled to room temperature. Once cooled, 2.0 ml of a chloroform:methanol:hexane (1:1:78, v/v/v) solution containing 0.25 mg each of stearic and oleic acid was added. To this mixture, 0.1 ml of concentrated HCl was added. After acidification, 1.0 ml from the upper, hexane, layer was removed. The remaining solution was reextracted four times by adding 1.2 ml of hexane, mixing (vortex mixer), and withdrawing 1.0 ml of the hexane layer. All of the hexane layer aliquots were combined. The hexane solutions were then evaporated to dryness under a stream of N_2 with slight warming. The lipid residue was redissolved in 0.3 ml of chloroform:methanol (2:1, v/v). Methanolic HCl (10 %) was added to the tubes and the mixture was agitated and kept at room temperature for one hour. The methanolic HCl step served to methylate any remaining free fatty acids (25). The samples were again dried with N_2 and slight heat. The dried samples were treated in the same manner as the methyl esters of the ammonium stearate systems.

RESULTS

The enzyme activity was expressed as percent conversion of substrate, which was calculated by dividing the methyl oleate counts per minute by the sum of the methyl oleate and methyl stearate counts per minute and multiplying this value by 100. The number of picomoles of product, methyl oleate, formed was obtained by multiplying the degree of conversion by the number of picomoles of substrate at the start of the assay. To expedite comparisons among the assays, some data are reported as normalized data. To normalize, the activity for each control was arbitrarily expressed as 100. The values presented for the other tubes of a given assay are their conversions relative to the control. The raw data that served as the source of the normalized data presented in this report appear in the appendix.

Table I presents the dependence of the ammonium stearate substrate assay on required cofactors.

The relationship between the activity of the desaturase complex, when ammonium stearate was the substrate, and incubation time is presented in Figure 1. It can be seen that there is very little oleate formation during the first 5 min of incubation. Following this lag, there is an increase in the rate of oleate formation. Between 9 and 13 minutes, the rate of oleate formation appears to be nearly constant, resulting in an approximately linear product-time relationship. Times longer than 20 minutes were not used as Paulsrud et al (21) and Raju and Reiser (26) suggested that beyond this point no further oleate was formed. In agreement with this finding of Raju and Reiser (26) oleate formation in one experiment appeared to level off after 15 min.

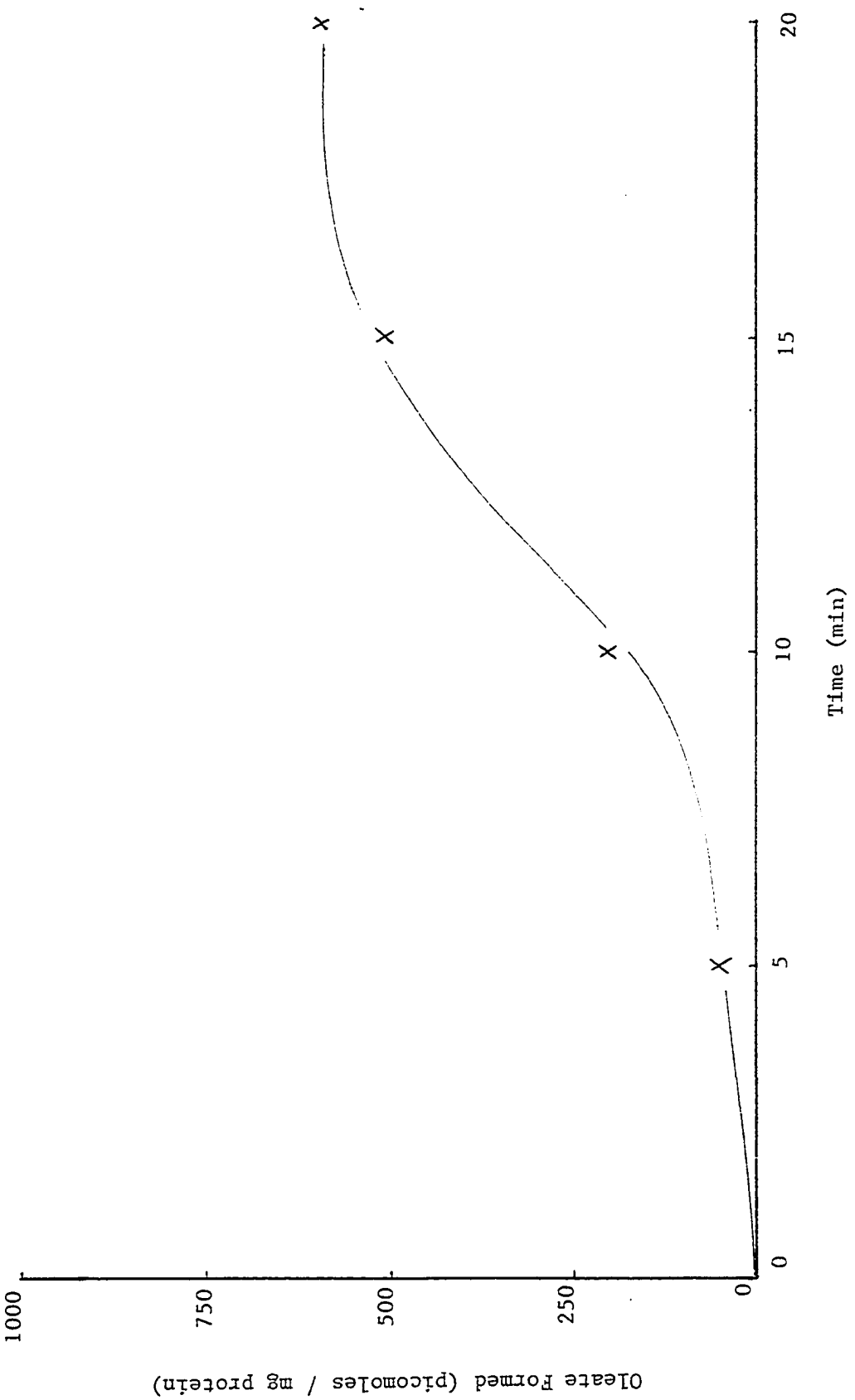
Table I

Effect of Cofactors on Desaturase Activity

(Assay System II)

Assay Mixture	Relative Activity
Complete	100
less NADH	30
less ATP	36
less CoA	26
less NADH, ATP, and CoA	27
less Enzyme	23

Figure 1. Effect of Incubation Time on Oleate Formation: Ammonium Stearate Substrate; Assay System II



The entire time course of the reaction appears to form an S-shaped curve with an inflection point at about 11 min.

The variation of the activity of the desaturase complex as a function of the quantity of microsomal protein is presented in Figure 2. As can be seen, the amount of stearate desaturated was a linear function of the protein present.

The time course of the reaction using stearoyl-CoA as substrate, was also S-shaped (Figure 3). In this assay, the rate of oleate formation appeared to be nearly constant between 11 and 15 minutes. This rate is represented in the figure as a linear region and suggests an inflection point at 13 min. After 20 min, oleate production leveled off and no further significant increase was observed over the next 20 min.

As shown in Figure 4, there is an approximately linear relationship between the amount of protein present and the quantity of oleate formed when stearoyl-CoA was used as substrate. The greatest percent deviation occured at the lowest protein concentration. The assays using the metabolites had between 5.0 and 6.3 mg protein present. With this amount of protein the deviation is $\pm$ 10 percent.

The percent conversion of stearate to oleate by the desaturase complex with and without a 10 mM concentration of metabolite is presented in Table II. The relative deviations of the controls and those of the experimental tubes are indicated. The combined deviation represents the square root of the sum of the squares of the relative deviations. The last column shows the percent change in the conversion of the experimental tubes over that of the controls using the

Figure 2. Effect of Microsomal Protein Concentration on Oleate Formation: Ammonium Stearate Substrate; Assay System III

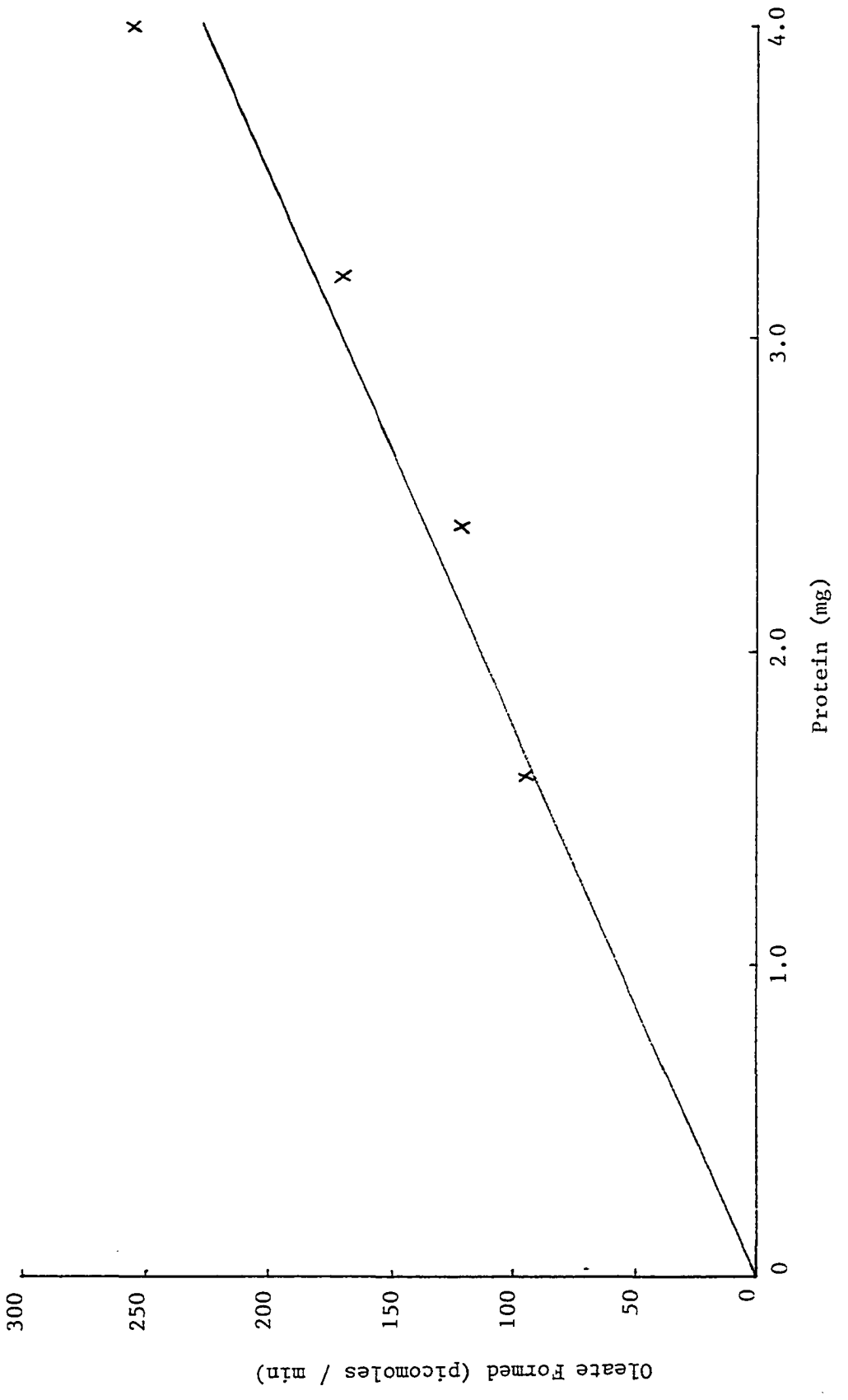


Figure 3. Effect of Incubation Time on Oleate Formation: Stearoyl-CoA Substrate; Assay System IV

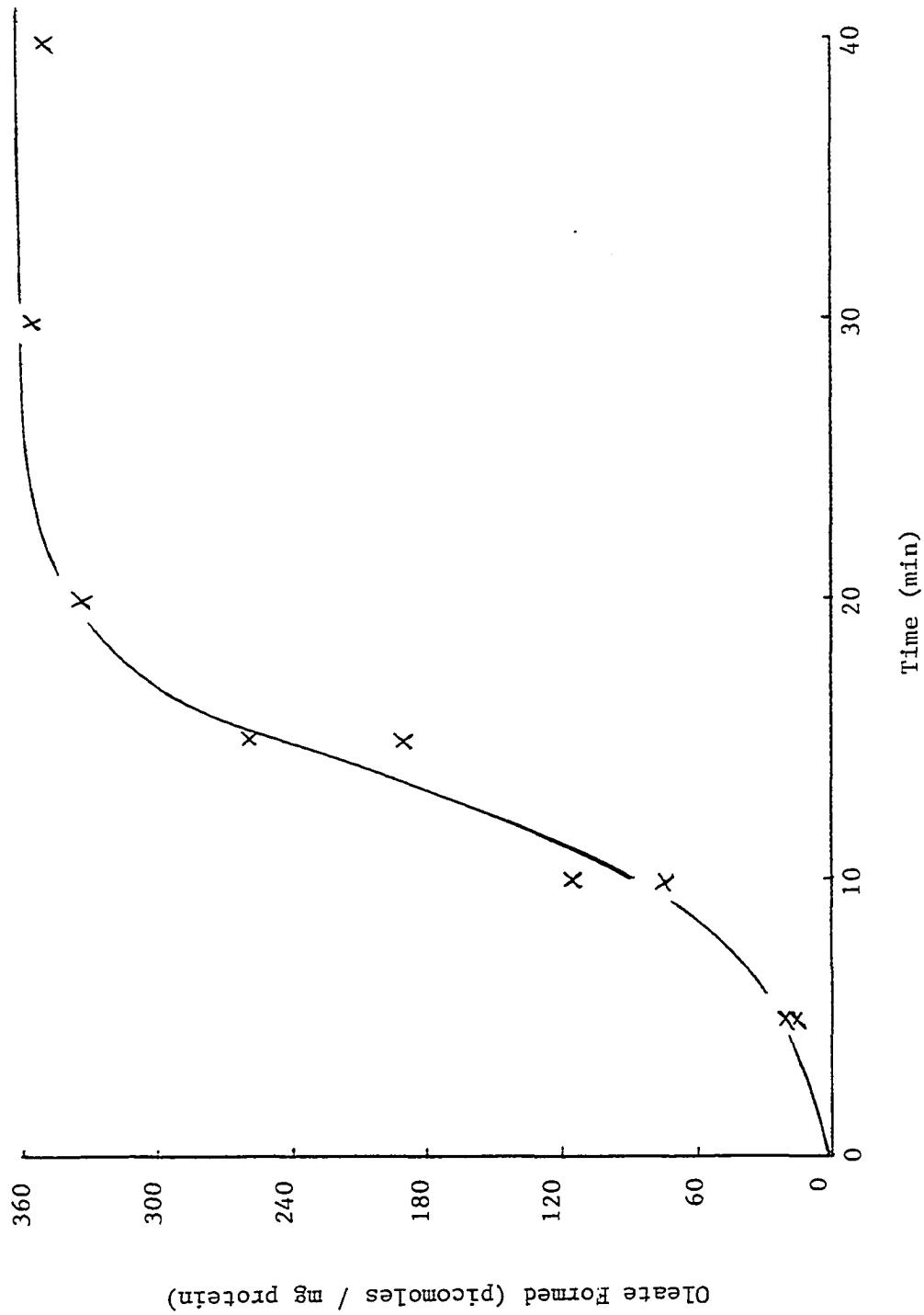


Figure 4. Effect of Microsomal Protein Concentration on the Oleate Formation: Stearoyl-CoA Substrate; Assay System IV

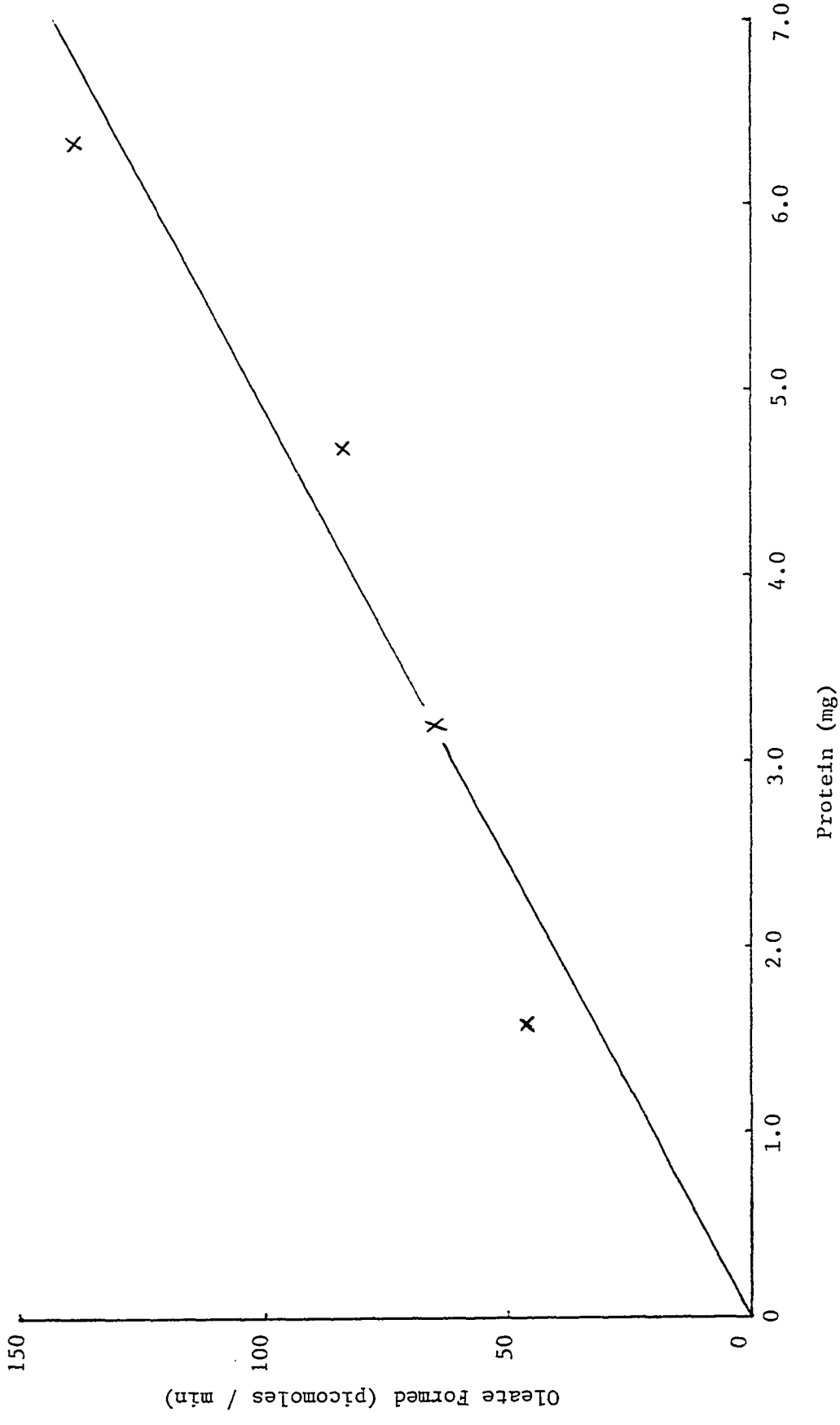


Table II

Percent Conversion of Stearate to Oleate by the Desaturase Complex:
Assay System I

(10 mM Metabolite Concentration)

metabolite	control		relative deviation	experimental		relative deviation	combined deviation	percent change
phenylethylamine	1.32	0.97	15.8	1.49	1.58	5.8	16.8	+35
o-hydroxyphenylacetic acid	1.52	1.29	8.6	0.99	1.21	10.0	13.2	-22
o-tyrosine	1.39	1.53	4.8	1.33	0.79	25.5	25.9	-27
phenyllactic acid	1.28	1.40	4.5	1.30	0.87	20.4	20.9	-30
norepinephrine	2.30	2.37	0.4	2.06	2.34	6.4	6.4	- 7
epinephrine	2.01	1.45	16.2	0.54	0.54	0.0	16.2	-90
phenylpyruvic acid	2.20	1.53	18.3	2.90	2.03	17.9	25.6	+55
L-DOPA	1.03	0.96	7.0	0.63	0.78	11.4	13.4	-37

average for each set of tubes. These are presented graphically as the normalized activities in Figure 5.

A metabolite was considered to produce a change in the activity of the complex if: a) the range of the controls did not overlap that of the experimental tubes and vice versa, and b) the change was more than the combined deviation. Using these criteria, o-hydroxyphenylacetic acid, o-tyrosine, epinephrine, and L-DOPA were all considered to be inhibitors and phenylethylamine was considered to be an activator.

Table III and Figure 6 present results of similar experiments using the metabolites at a 0.1 mM concentration. Following the above criteria, phenyllactic acid, and o-hydroxyphenylacetic acid were considered to be inhibitors. As the combined deviation could not be calculated in several cases, the criteria were changed for these cases, requiring part (a) and a difference greater than 20 percent between the controls and experimental tubes. Using these revised criteria, phenylpyruvic acid is considered to be an inhibitor and phenylethylamine and tryptamine are considered to be activators.

The activity of the desaturase complex using stearoyl-CoA as the substrate, in the presence of various concentrations of phenyllactic acid is given in Figure 7. From these data it can be seen that phenyllactic acid is an inhibitor of the desaturase complex over the concentration range of 53 to 165 μ M. Additionally, the desaturase activity in the presence of phenyllactic acid and using ammonium stearate as the substrate (Figure 6), falls within the general area of the corresponding points using stearoyl-CoA as substrate (Figure 7).

Figure 5. Desturase Activity in the Presence of 10 mM Concentrations of Metabolites: Ammonium Stearate Substrate; Assay System I

A: Control

B: Phenylethylamine

C: o-Hydroxyphenylacetic acid

D: o-Tyrosine

E: Phenyllactic acid

F: Norepinephrine

G: Epinephrine

H: Phenylpyruvic acid

I: L-DOPA

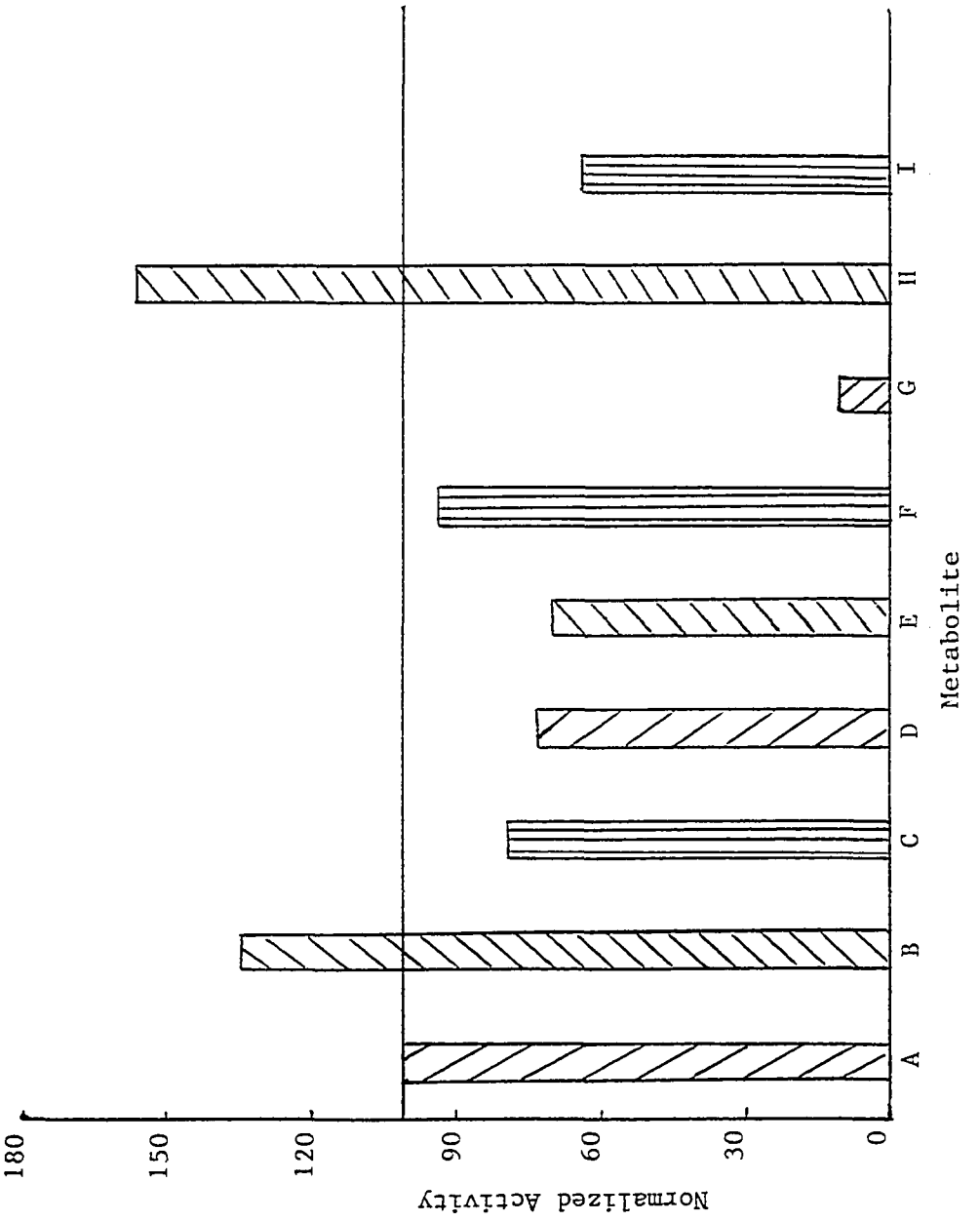


Table III

Percent Conversion of Stearate to Oleate by the Desaturase Complex:
Assay System I

(0.1 mM Metabolite Concentration)

metabolite	control		relative deviation	experimental		relative deviation	combined deviation	percent change
phenylethylamine	1.59	---		2.17	1.84	8.5	---	+38
o-hydroxyphenylacetic acid	1.72	1.78	1.7	1.55	1.69	4.3	4.6	- 9
o-tyrosine	1.72	1.78		1.60	2.13	14.5	14.6	+28
phenyllactic acid	4.97	3.26	20.9	2.27	2.55	5.8	21.7	-45
norepinephrine	1.53	1.61	2.5	1.55	1.22	12.3	12.6	-14
epinephrine	1.53	1.61		1.52	---		---	- 4
phenylpyruvic acid	1.85			1.51				-18
	5.28			3.88				-27
L-DOPA	4.97	3.26		2.85	3.70	12.8	24.5	-22

Table III

(Continued)

metabolite	control		relative deviation	experimental		relative deviation	combined deviation	percent change
phenylacetic acid	4.97	3.26		3.39	5.66	25.2	32.7	+18
tryptamine	1.59	---		2.06	1.70	9.6		+26
2-aminophenylethanol	5.33	5.61	3.1	5.37	4.73	6.3	7.0	-10
phenylethanol	5.33	5.61		5.55	3.04	29.3	29.5	-27
indolephyruvic acid	1.85			1.61				-13
	5.28			5.12				- 3
phenylalanine	4.97	3.26		4.14	3.06	15.0	25.7	-14

Figure 6. Desaturase Activity in the Presence of 0.1 mM Concentration of Metabolites; Assay System I

A: Control

B: Phenylethylamine

C: o-Hydroxyphenylacetic acid

D: o-Tyrosine

E: Phenyllactic acid

F: Norepinephrine

G: Epinephrine

H: Phenylpyruvic acid

I: L-DOPA

J: Phenylacetic acid

K: Tryptamine

L: 2-Aminophenylethanol

M: Phenylethanol

N: Indolepyruvic acid

O: L-Phenylalanine

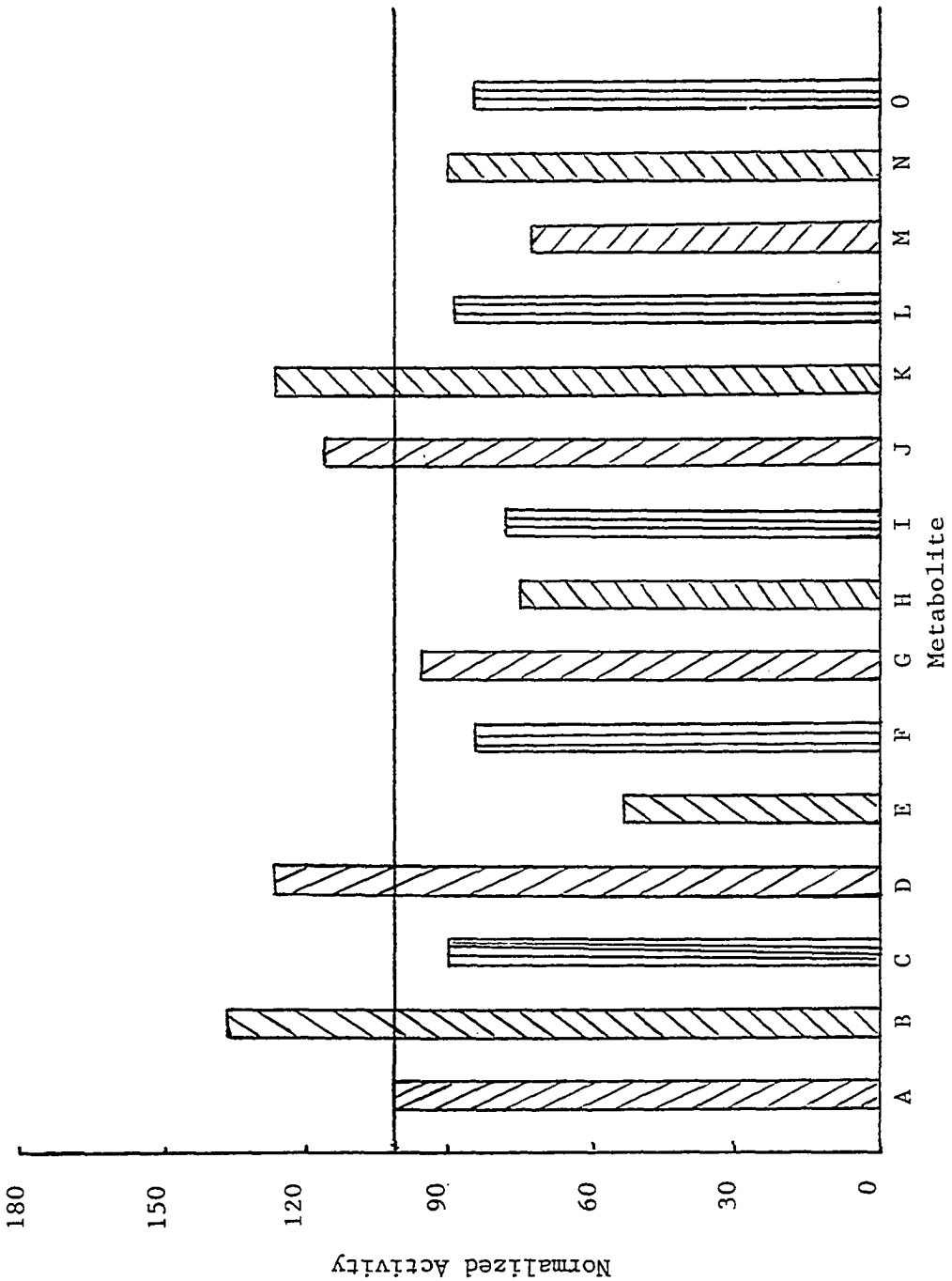
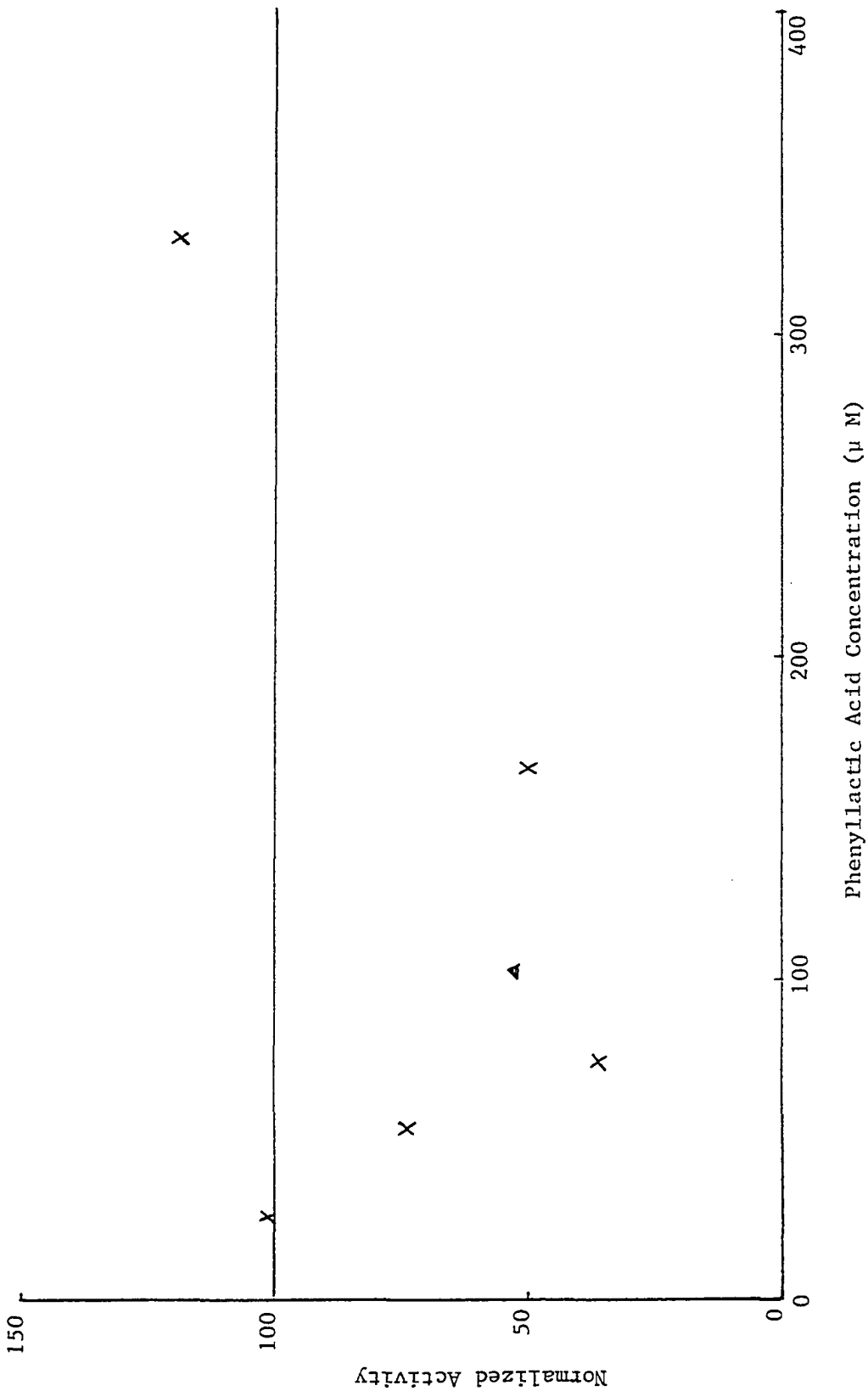


Figure 7. Effect of Phenyllactic Acid Concentration on Desaturase Activity

✕ Stearoyl-CoA Substrate, Assay System IV

▲ Ammonium Stearate Substrate, Assay System II



As can be seen from Figure 8, the activity of the desaturase, using stearoyl-CoA as the substrate, appears to change from a 30 percent inhibition to a 30 percent activation as the epinephrine concentration was increased from 100 to 400 μ M. Further increases in the epinephrine concentration resulted in lower activities. The decreasing activity trend shown in Figure 8 continues as far as concentrations of 10,000 and 40,000 μ M. (see Appendix)

The activity of the desaturase complex, using stearoyl-CoA as the substrate, in the presence of several concentrations of phenylacetic acid is shown in Figure 9. These data suggest that low (100 μ M) concentrations of phenylacetic acid activate the enzyme complex. At 400 and 1000 μ M concentrations, the activation appeared to be progressively lost, but activation returned at 4000 μ M.

Figure 8. Effect of Epinephrine Concentration on Desaturase Activity

X Stearoyl-CoA Substrate, Assay System IV

▲ Ammonium Stearate Substrate, Assay System II

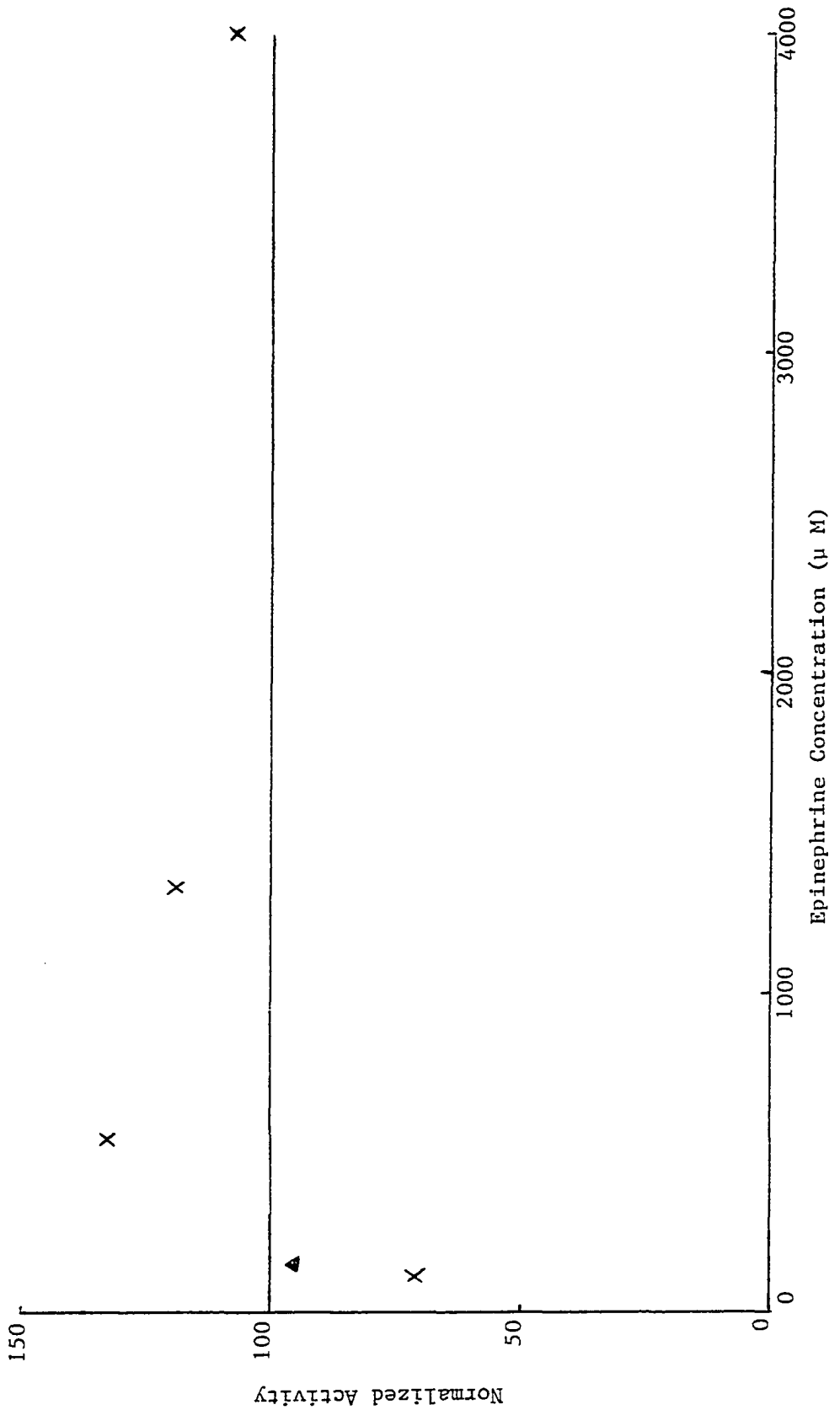
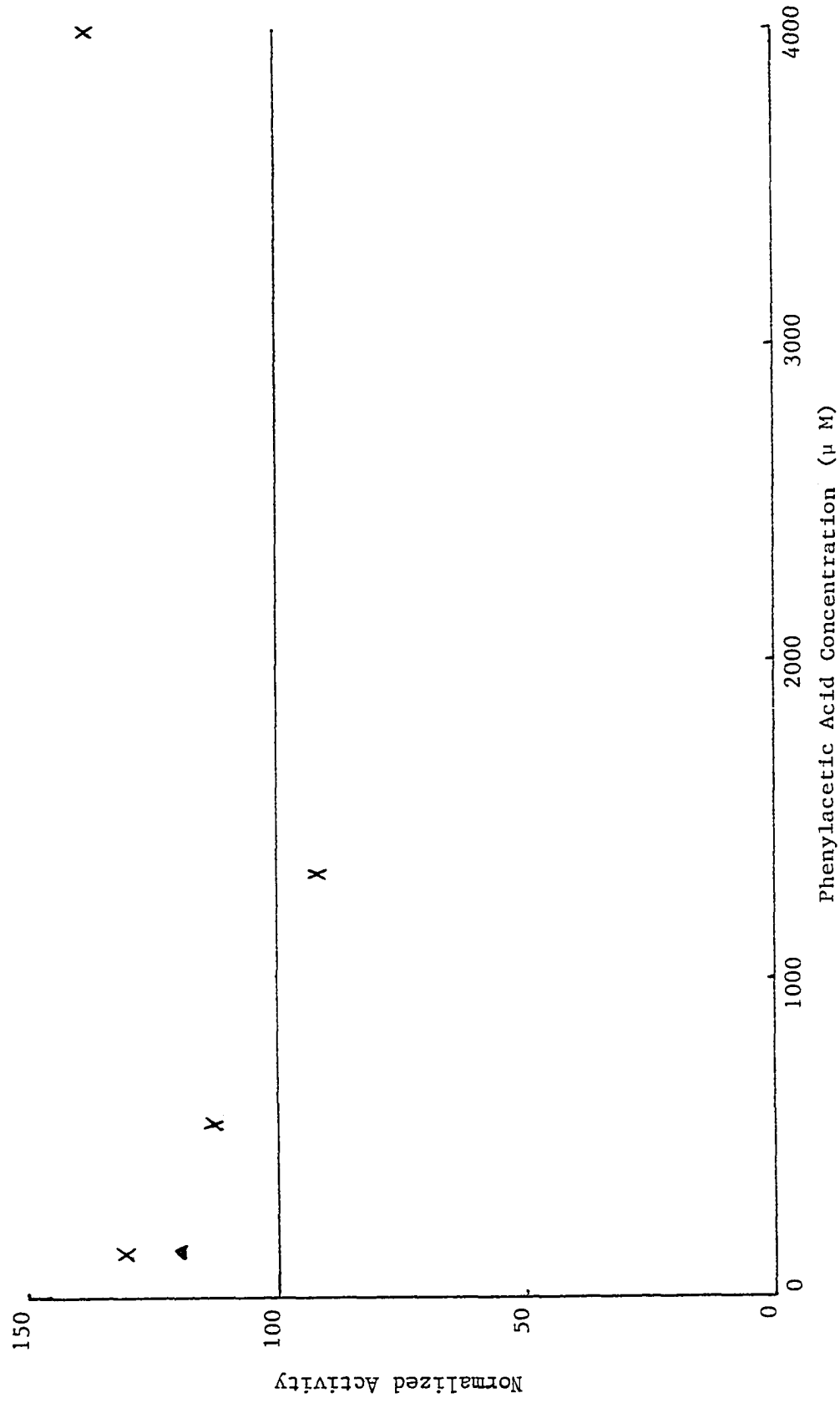


Figure 9. Effect of Phenylacetic Acid Concentration on Desaturase Activity

✗ Stearoyl-CoA Substrate, Assay System IV

▲ Ammonium Stearate Substrate, Assay System II



Discussion

Successive enzyme preparations were different. This difference largely reflects experience in isolating the microsomes and is best illustrated by the variation in protein concentration in the various enzyme preparations. The protein concentration of these preparations ranged from 0.6 to 25.9 mg per ml. The preparations, which were used for all the reported assays contained between 4.0 and 7.9 mg protein per ml of microsome solution.

The major reason for using ammonium stearate in the screening assays was the cost of ammonium stearate compared tc that of stearoyl-CoA. The ammonium salt was chosen, following the suggestion of Lands (27), after finding that neither free stearic acid nor the sodium salt was sufficiently soluble (28). Once the metabolites had been screened, it was felt that further data would better reflect inhibition of the desaturase if stearoyl-CoA were used a substrate.

The stearic acid and oleic acid isolated after incubation amounted to about 30 % recovery of the radioactivity used in the assay. The incomplete recovery was probably due to the association of the hydrophobic microsomal proteins (12, 13) with the organic solvent molecules, as well as to the absorption of a certain portion of the stearate and oleate on the microsomal proteins (29). To minimize the effect of these losses, a large non-radioactive excess of each acid was added before the extractions. Since the recovery of radioactivity was not uniform, the amount of oleate formed was calculated as a percentage of the total oleate and stearate recovered, as previously was

described. Variations in duplicate assays were in part due to incomplete removal of the methyl esters from the TLC plates.

As a consequence of the linearity of the plots of protein versus activity (Figure 2, 4) these assay systems appear to be useful for the detection of inhibitors of the desaturase complex.

A complication in the assays in which ammonium stearate was used as the substrate is the necessity to activate the stearate to stearoyl-CoA by the following reaction (30) which is catalyzed by the enzyme CoA-ligase:

$$CoA + ATP + R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O^{-} = AMP + PP + R\text{-}\overset{\overset{O}{\|}}{C}\text{-}S\text{-}CoA + OH^{-}$$

Ammonium stearate is not an effective substrate for the desaturase complex (15). The ineffectiveness of ammonium stearate as the substrate is demonstrated in Table I. Incubations lacking either ATP or CoA showed no significant activity when ammonium stearate was the substrate. Both species are required for the activation of the ammonium stearate. Paulsrud et al (21) and Pande and Mead (31) state that the acyl-activation is not the rate limiting step in the overall desaturation system.

Oshino, Imaim, and Sato (14) suggest that the leveling off of oleate formation with time is the result of the irreversible consumption of stearoyl-CoA by the several enzymes present in the microsomal preparation. Most microsomal protein preparations contain acyl-CoA hydrolases, acyl transferases (32, 33), and acyl elongation enzymes (34). These competing enzymes probably are present in different

amounts in different microsomal preparations which may account for some of the variation in the measured activity of this desaturase.

The concentration of metabolites in the first survey was arbitrarily chosen. Many of the metabolites were not sufficiently soluble to allow their use at a 10 mM concentration and were, therefore, used at a 0.1 mM concentration. Additionally, the survey with lower metabolite concentrations corresponds better to the substrate concentration. Furthermore, according to Menkes, phenylpyruvic acid is 0.2 mM in the tissue of untreated phenylketonurics (9).

The inhibition of the desaturase by phenyllactic acid is consistent with the general hypothesis mentioned in the Introduction. Brains from rats having experimentally induced phenylketonuria had low oleic-stearic acid ratios when the rats were 25 and 31 days old (11). This follows the period of rapid myelination which is generally considered to be impaired in phenylketonuria. During the period of rapid myelination it has been shown that the conversion of phenylalanine to phenyllactic acid increases (35). Also, phenyllactic acid crosses the blood-brain barrier (35). Therefore, during the period of rapid brain development in a phenylketonuric, the phenylalanine concentration is high and at the same time it is being converted to phenyllactic acid at an increased rate. The phenyllactic acid then inhibits the conversion of stearate to oleate and hence the observed reduced ratios between these two acids. Whether or not this reasoning can be extrapolated to explain the lowered ratio found in human phenylketonurics (8) is unknown. The inhibition of the desaturase by 0.1 mM phenylpyruvic acid is consistent with the same argument.

Assuming that the usual interpretation of kinetic data for an enzyme with a soluble substrate could be made, the data were analyzed in the form of a Lineweaver-Burk plot. However, the plot obtained was originally very perplexing, as it had a negative slope. The fact that palmitoyl-CoA, an analog of stearoyl-CoA, has a critical micellar concentration between 4 and 8 μ M (36) suggest an explanation of the negative slope. Assuming that stearoyl-CoA forms micelles, similar to those formed by palmitoyl-CoA, there would be a constant concentration of molecular stearoyl-CoA at all stearoyl-CoA concentrations greater than the critical micellar concentration.

The plots of oleate formation as a function of time showed an S-shaped pattern. The tailing of the curve at long incubation times is presumably due to the low concentration of substrate as suggested by Oshino et al (14). Before the leveling off of the curve the rate of oleate formation increases with time. Since substrate concentration decreases with time, it appears that the rate of oleate formation increases as a function of decreasing substrate. Several authors have reported that stearoyl-CoA is an inhibitor of stearoyl-CoA desaturase (31, 32) and the present results are in line with those reports.

Baker and Lynen (29) reported that stearoyl-CoA is tightly bound by, but not covalently linked to, microsomal protein. These authors gave no indication whether there is preferential binding for the molecular or the micellar form of stearoyl-CoA. It has been assumed here that both forms are bound approximately equally.

Gatt and Bartfai (37) have simulated a velocity versus substrate

concentration plot, and hence a Lineweaver-Burk plot, for an enzyme that has: (1) a "soluble" lipid substrate which forms micelles; (2) a reactivity preference for the molecular substrate; and (3) approximately equal binding of both the molecular substrate and the micelles at the active site of the enzyme. In this simulated plot, the reaction velocity increases as a function of substrate concentration until the critical micellar concentration is reached. Above the critical micellar concentration, further increases in the substrate concentration increases in the substrate concentration increase the micellar concentration, but result in a decrease in the reaction velocity. The resulting Lineweaver-Burk plot is V-shaped with the minimum at the inverse of the critical micellar concentration.

For the stearoyl-CoA desaturase system, Gatt and Bartfai's model is an oversimplification. The observed critical micellar concentration is altered by the adsorption of the substrate by the microsomal proteins at other than the catalytic site. This means that a thorough treatment of the Michaelis-Menten kinetic theory for the present case should incorporate the Langmuir isotherm into the substrate concentration term. As a result of the complexity in interpretation of this system, the kinetic data obtained were inadequate for a meaningful Lineweaver-Burk interpretation.

In considering the hypothesis that the alteration of the oleic-stearic acid ratio observed is the result of inhibition of the stearoyl-CoA desaturase complex, it is useful to examine an implication of the hypothesis. Foote, Allen, and Agranoff (8), in addition to finding an inversion in the oleic-stearic acid ratio for the total

esterified lipids of a phenylketonuric brain compared to a nonphenylketonuric brain, found that the ratio of the 24 carbon monoene fatty acid to the saturated 24 carbon fatty acid from cerebrosides and cerebroside sulfates changed in the same manner as the 18 carbon fatty acids did. The monoene to saturated fatty acid ratio decreased. As stearic acid elongation is the most probable source of the saturated 24 carbon fatty acid and oleic acid elongation is the most likely source of monoene 24 carbon fatty acid (38, 39, 40), the inhibition of stearoyl-CoA desaturase as a plausible explanation of both effects is appealing due to its simplicity it requires only one change in the cell function.

It is interesting to note that the inhibitors found in the 10 mM metabolite survey do not correspond to those found in the lower concentration survey. The high concentration probably does not correspond to physiological conditions. Furthermore, it is very possible that these metabolites, as well as the metabolites found to have an effect at the lower concentration, do so by altering the micellar concentration. Another point about the metabolites having an effect at the lower concentration is that the two activators were aromatic amines and the three inhibitors were aromatic acids.

Alternative hypotheses to explain the low oleic-stearic acid ratios include: differential absorption in the small intestine of oleic and stearic acids; differential absorption of oleic and stearic acids by brain cells; and differential incorporation into the fatty acid pool of brain cells of these acids. The second and third possibilities differ in that the third may reflect a difference in the

incorporation into glycerolipids between stearic and oleic acids. Other possibilities for the lowered ratios include an altered preference in the formation or the hydrolysis of the acyl-CoA esters, or in the degradation of the respective acyl-ester. Some metabolic abnormality must be causing the low oleic-stearic acid ratio which overshadows the relatively high amounts of oleic acid in the diet. The ratio of oleic to stearic acid in a baby's diet is between 4.5 in human milk (41) and 5.0 in rat chow (42).

Another explanation of the alteration in the oleic-stearic acid ratio comes from an interpretation of data from Weber et al (43), who showed that phenylpyruvic acid inhibits human brain hexokinase. These same authors also reported that L-phenylalanine reduces pyruvate kinase activity from the same source. A probable result of these inhibitions in brain is a decreased concentration of the reduced dinucleotides NADH and NADPH. The possible loss of these nucleotides would affect the desaturation reaction as one or the other of these is a required cofactor. Furthermore, Weber et al (43) showed that the activity of these enzymes from fetal human brain is 10 percent of that from adult brain which would accentuate the effect. Moreover, Baker and Lynen (29) report that decreasing the level of the reduced dinucleotides resulted in a net hydrolysis of stearoyl-CoA.

Other studies have shown that the high levels of phenylalanine present in phenylketonuria may inhibit protein synthesis. Neame (44) and Linneweh et al (45, 9) have reported that the transport of L-amino acids into a number of tissues, amongst them brain, is inhibited by the presence of phenylalanine at the plasma concentrations present

in phenylketonuria. Moreover, Appel (46) demonstrated a 70 percent inhibition of in vitro synthesis of tyrosine tRNA when the ratio of phenylalanine to tyrosine was 100:1. In phenylketonuria this ratio is closely approached. Thus, protein synthesis may be reduced in phenylketonuria.

Finally, there are a number of other changes present in phenylketonuria (9), and while none of these individually has been shown to be responsible for the mental deficiency present in this disease, the effect of a composite of factors may go a considerable distance in explaining the observed mental retardation.

The findings reported here are consistent with the hypothesis that the lowered oleic-stearic acid ratio found in phenylketonuric brains results from an inhibition of desaturase. Specifically, it appears that phenyllactic acid at the tissue concentrations of an untreated phenylketonuric inhibits this complex. However, caution must be excercised in interpreting the data as an apparent inhibition may reflect the stearic acid and stearoyl-CoA aggregation instead of a direct effect upon the enzyme. Also the data presented do not permit a distinction between the direct inhibition of the desaturase by a metabolite, present in unusual amounts, and the indirect inhibition by phenylpyruvate on reduced dinucleotide formation.

APPENDIX

Effect of Incubation Time on Oleate Formation: Ammonium Stearate Substrate; Assay System II, see page 7

Time (min)	5	10	15	20
Percent Conversion	0.19	0.74	2.01	2.66

Effect of Microsomal Protein Concentration on Oleate Formation: Ammonium Stearate Substrate; Assay System III, see page 7

Enzyme (mg)	1.6	2.4	3.2	4.0
Percent Conversion	0.96	1.22	1.71	2.57

Effect of Incubation Time on Oleate Formation: Stearoyl-CoA Substrate; Assay System IV, see page 9

Time (min)	5	5	10	10
Percent Conversion	0.13	0.12	0.46	0.73

Time (min)	15	15	20	30	40
Percent Conversion	1.63	1.20	2.09	2.24	2.22

Effect of Microsomal Protein Concentration on Oleate Formation: Stearoyl-CoA Substrate; Assay System IV, see page 9

Enzyme (mg)	1.6	3.2	4.7	6.3
Percent Conversion	0.69	0.96	1.26	2.08

Effect of Phenyllactic Acid Concentration on Desaturase Activity: Stearoyl-CoA Substrate; Assay System IV, see page 9

Phenyllacitc Acid (μ M)	---	---	27	53	80	170	330
Percent Conversion	3.71	4.82	4.41	3.25	1.81	2.36	4.97

Effect of Epinephrine Concentration on Desaturase Activity: Stearoyl-CoA Substrate; Assay System IV, see page 9

Epinephrine (mM)	---	---	0.10	0.40	1.0	4.0	10.	40.
Percent Conversion	0.55	0.60	0.40	0.76	0.67	0.62	0.54	0.45

Effect of Phenylacetic Acid Concentration on Desaturase Activity: Stearoyl-CoA Substrate; Assay System IV, see page 9

Phenylacetic Acid (mM)	---	0.10	0.40	1.0	4.0
Percent Conversion	1.70	2.18	1.89	1.59	2.31

In all of the above cases the blank has been substracted out.

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