EXPERIMENTAL HYPERPHENYLALANINEMIA IN THE PREGNANT AND NONPREGNANT GUINEA PIG

By

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Abstract

Phenylketonuria (PKU) is an inherited disorder of amino acid metabolism in which the conversion of L-phenylalanine (Phe) to L-tyrosine (Tyr) is greatly diminished. This metabolic block results in high blood Phe levels with low to normal Tyr levels and is usually accompanied by mental retardation unless the disease is treated by dietary restriction of Phe early in life. In recent years it has become apparent that many of the non-PKU offspring of PKU women are damaged in utero by their mother's disease. Approximately 90% of the non-PKU children of PKU mothers are mentally retarded, over 50% are microcephalic and suffer intrauterine growth retardation, while nearly 40% have some congenital malformation. The risk to the offspring of women with hyperphenylalaninemia (hyperphe) probably decreases as the maternal Phe level decreases, althought the "safe" level of maternal hyperphe is not yet well defined. It does appear however, that the developing offspring of women with Phe levels of at least 15 mg/100 ml face substantial risk of in utero damage. Attempts to prevent offspring damage by treatment of pregnant hyperphe women with dietary restriction of Phe have had only limited success. There are still many unanswered questions regarding the management of maternal hyperphe and these will become even more pressing as increasing numbers of PKU women who were success-

fully treated as children reach reproductive age. Since clinical data are limited attempts have been made to establish experimental animal models of maternal hyperphe.

Early animal studies produced maternal hyperphe by administering large doses of Phe to pregnant animals. Although some of these studies suggested associations between maternal hyperphe and behavioral and biochemical abnormalities found in the offspring, most were confounded by maternal hypertyrosinemia, an abnormality never found in PKU. More recent approaches have utilized the Phe hydroxylase inhibitor p-chlorophenylalanine (pCPA) in order to prevent Tyr elevation following Phe administration. Experiments using both Phe and pCPA administration have more closely mimicked the biochemical characteristics of PKU, however methodological problems have limited the interpretation of many of these studies. In addition, the work with combined Phe and pCPA treatment has been restricted to the rat, a species in which significant differences from human prenatal development exist. The prenatal development of the guinea pig is more similar to man, especially with respect to the brain, and a study was therefore undertaken to determine the suitability of the guinea pig as a possible model of maternal hyperphe. Initially guinea pigs were injected with various regimens of Phe and pCPA. Blood Phe was transiently elevated to levels comparable to those found in PKU patients, however even following very high doses of both Phe and pCPA, Phe fell to near-normal levels

within 10 hours of the Phe injection. The blood Phe response of the animals injected with pCPA suggested that the Phe hydroxylase inhibition induced by pCPA in guinea pigs is of a much shorter duration than has been reported in rats. Since Phe does not fall to normal levels at any time in untreated PKU, other methods of administering pCPA and Phe were evaluated. Studies were undertaken in which both Phe and pCPA were incorporated into test diets and then fed to guinea pigs. This approach resulted in hyperphe comparable to that associated with significant risk to the human fetus which could be maintained for many weeks in both pregnant and nonpregnant animals. The optimum dietary pCPA supplement was determined by maximizing hepatic Phe hydroxylase inhibition and plasma Phe concentration. The amount of pCPA required to maximally decrease Phe hydroxylase activity in guinea pigs is considerably more than is needed in the rat. Indirect evidence was obtained which suggests that this species difference may be due, at least in part, to rapid excretion of pCPA as p-chlorophenylpyruvic acid by the guinea pig. The appropriate dietary Phe supplement was determined by monitoring plasma Phe and Tyr levels as well as food intake and weight gain in animals fed test diets supplemented with pCPA and various amounts of Phe. Additional work demonstrated that plasma Phe levels remained elevated for at least a 12 hour period during a single day and that ascorbic acid is needed for guinea pigs to efficiently metabolize Tyr.

When stable maternal hyperphe was induced by feeding

pregnant guinea pigs appropriate test diets, abortion occurred and was found to be related to pCPA, even in the absence of substantial hyperphe. Further study of the effects of pCPA and hyperphe during early pregnancy was undertaken by feeding guinea pigs test diets from day 1 of pregnancy and collecting embryos on gestation day 17. Only pCPA was associated with embryonic death, however malformed embryos were significantly associated with maternal hyperphe, even in the absence of pCPA administration. The relationship between maternal hyperphe and malformed embryos had not been previously demonstrated in animals and it may have relevance to the high frequency of congenital defects found in offspring of PKU women. Evidence of embryonic developmental retardation was also found and hyperphe may be causally related to this abnormality as well. Both Phe and Tyr were found to be actively transported to the early embryo and this transport of Phe might be related to its teratogenicity. The embryo toxicity of pCPA limits the utility of the current approach. Use of newer Phe hydroxylase inhibitors in pregnant guinea pigs may prove informative.

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Introduction

The Clinical Problem

Phenylketonuria (PKU) is an inherited disorder of amino acid metabolism characterized by a diminished ability to convert L-phenylalanine (Phe) to L-tyrosine (Tyr) resulting in abnormally high blood Phe levels. Normally the blood Phe concentration is below 2 mg/100 ml whereas in classical PKU it exceeds 20 mg/100 ml. Early detection and dietary restriction of Phe is effective in preventing the mental retardation usually found in untreated patients (Scriver and Rosenberg 1973; Berry 1976). Once the developing brain has passed the period of sensitivity to the toxic effects of Phe, dietary treatment of children with PKU is terminated, usually during the early school years. Upon resumption of a normal diet, Phe returns to the abnormally high levels characteristic of PKU. Since 1963 however, a new aspect of this disease has been revealed. In that year a number of reports appeared which described mental retardation and early infant death in the non-PKU infants born to PKU women (Denniston 1963; Mabry et al 1963). Subsequent cases were promptly reported which expanded the number of effects attributed to maternal PKU to include not only mental retardation but also microcephaly, prenatal and postnatal growth retardation, congenital malformations and spontaneous

abortion (see references in Tables 1 through 5). Over 280 pregnancies in women with hyperphenylalaninemia (hyperphe) have already been reported. Many successfully treated PKU children are just now reaching reproductive age and the number of PKU women becoming pregnant is therefore expected to increase dramatically in the coming years.

Since physicians are being faced with increasing numbers of PKU women who are considering pregnancy or who are already pregnant, it is important to clearly define the risks associated with maternal hyperphe. All available reports of maternal hyperphe were therefore reviewed in order to estimate the magnitude of these risks. Tables 1, 2, and 3 show the effects of classical PKU (defined as a blood Phe level greater than or equal to 20 mg/100 ml) on the pregnancies of over 50 women. Since many cases of maternal PKU have been ascertained because of an affected infant, hence biasing the data, the cases reviewed have been tabulated on the basis of the method of ascertainment. Table 1 includes the cases which were discovered because of an affected child, the criterion used for defining biased ascertainment in this review. Table 2 includes the unbiased data defined as those cases in which the family came to medical attention for reasons other than the presence of affected offspring. Table 3 illustrates the data for which the means of ascertainment was not reported. In the past, biased data may have exaggerated the estimated risk to the

Table 1 Classical phenylketonuria*: Outcome of untreated cases ascertained because of affected offspring (Biased)

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations Comments
Mabry etal 1966	22-26	97	0	88	-	0	0	0
				70-80	-	0	0	seizures
				80-85	-	0	0	0
				85	-	0	0	0
	26-28	92	0	74	-	0	+	0
				77	-	0	0	0
				56	-	0	+	0
				75	-	0	0	0
Fisch etal 1966, 1969	26	88	1	25-30	+(-)	+	+	seizures
				50-60	-	0	+	scoliosis
Frankenburg etal 1968	28-32	77	1	45	-	+	+	cleft eyelids, hemivertebrae, clubbed foot, seizures
				76	-	0	+	0
	19-34	74	2	54	-	0	?	0
				42	-	+	+	absent distal phalanx
				46	-	0	+	0
				66	-	+	+	0
Boucharlat etal 1972	21	63	0	64	-	+	+	strabismus
				88	-	+	+	0

Continued...

Table 1 (Con't.) Classical phenylketonuria*: Outcome of untreated cases ascertained because of affected offspring (Biased)

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations Comments
Williams 1968	23	62	0	51	-	+	?	CHD
				<50	-	?	?	0
				33-55	-	0	?	CHD
				56-72	-	?	?	0
Yu and O'Halloran 1970	26-31	"borderline"	2	N	-	?	0	0
				70	-	?	+	strabismus
				50	atypical (?)	?	+	strabismus
				?	atypical (?)	0	+	strabismus
	28	"retarded"	0	retarded	-	?	0	strabismus, seizures
				retarded	-	?	0	strabismus, hypertelorism, seizures
				retarded	-	?	0	seizures
				retarded	-	?	0	strabismus
				retarded	-	?	+	seizures
				retarded	-	?	+	seizures
Montenegro etal 1965	25	N	0	?	?	?	?	CHD, died
				?	?	?	?	CHD, died
				?	?	?	?	CHD, died
				62	-	0	0	CHD
				53	-	0	0	0
				64	-	0	0	0

Continued...

Table 1 (Con't.) Classical phenylketonuria*: Outcome of untreated cases ascertained because of affected offspring (Biased).

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU ($\pm R_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations Comments
				50	-	0	0	0
				50	-	0	0	0
Hooft etal 1970	36	70	1	retarded	-	0	?	? died infection
				50	-	0	0	0
				55	-	0	0	0
				retarded	-	0	+	0
Gaudier etal 1972	28	80	8	retarded	-	+	+	0
				retarded	-	+	+	0
				retarded	-	+	+	club foot
MacCready and Levy 1972	"PKU"	N	?	retarded	-	?	?	0
				retarded	-	?	?	0
Levy etal 1968				retarded		?	?	0
Allan and Brown, 1968	27 (additional pregnancy treated with diet)	66	0	56	-	0	0	congenital cataracts EEG
Allan 1972 cited by MacCready and Levy 1972				88	-	0	0	congenital cataracts
				52	-	+	0	congenital cataracts
				"reduced intelligence"	-	?	?	?

Continued...

Table 1 (Con't.) Classical phenylketonuria*: Outcome of untreated cases ascertained because of affected offspring (Biased)

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ ♀ ♂	PKU (+R$_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
				"reduced intelligence"	-	?	?	?
Partington 1962	21	102	3	15	+(-)	+	0	partial syndactly
				103	-	?	0	0
				75	+ (+ from 3 mo.)	0	+	0
Perry and Tischler 1966	17-22	>90	0	41	+ (+ from 11 mo.)	?	+	0
Trouche etal 1974	19-28	66	0	?	?	+	?	CHD, died at 8 mo.
				37	-	+	+	0
				42	-	+	+	EEG
				37	-	0	+	0
				46	-	+	+	0
Huntley and Stevenson 1969	16-21	99	6	?	?	+	+	CHD, died at 2 days
				?	?	+	+	CHD(?) died at 8 days
Stevenson and Huntley 1967				38	-	+	+	EEG

Continued...

Table 1 (Con't.) Classical phenylketonuria*: Outcome of untreated cases ascertained because of affected offspring (Biased)

AUTHOR	MOTHER			OFFSPRING			
	Phe mg/100 ml	IQ Abortions	IQ/DQ	PKU ($\pm R_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
			?	-	0	+	CHD, hydrocele died 8 mo.
			?	?	0	+	Died at 2 wks. (infection?)

* maternal phenylalanine ≥ 20 mg/100 ml

Abbreviations:

- CHD: congenital heart disease
- DQ: developmental quotient
- EEG: EEG abnormal
- IQ: intelligence quotient
- IUGR: intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
- mo: month
- N: normal
- R_x: treatment
- wk.: week
- +: present
- 0: absent
- -: none
- ?: unknown

Table 2 Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased).

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU ($\pm R_x$) :	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Bovier-Lapierre etal 1974	15-29	73	1	50	-	+	+	died at 6 mo. facies, CHD, vertebrae myelin↓
Coffelt 1964	20-30	96	0	78	-	+	?	0
Forbes etal 1966				78	-	+	?	0
DeMenibus etal 1967	14-30	75 - 89	0	?	-	?	?	?
				?	-	?	?	?
				retarded	-	0	+	0 seizures
				twins ?	-	+	+	meningocele, died at 1 mo.
				twins ?	?	?	?	?
				twins 50	-	+	+	0 (C.F.?)
				twins ?	-	+	+	CHD, died (C.F.?)
				39	-	+	+	pyloric stenosis, seizures
Dennisten 1963	27	49	0	?	?	?	?	? died in infancy
Mabry etal 1963				?	?	?	?	? burned to death, 4 yr.
				30	?	?	?	?
				33	-	?	?	0

Continued...

Table 2 (Con't.) Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased)

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (+R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
				28	-	?	?	0
				61	-	?	?	0
				57	?	?	?	?
	26	82	0	?	?	?	?	? died at 1 day,
	(sister of above pt)			?	?	?	0	died 18 mo., "fever".
				30	-	?	?	0
				?	?	?	?	0 died 1 yr. pneumonia
				?	?	?	?	0 died at 1 mo.
				?	?	?	?	? died at 4 mo.
				?	?	?	?	? "died early"
				?	?	?	?	? died 6 yr. MVA
	26	27	0	24	-	?	?	0
Mabry etal 1966	30	52	0	49	?	?	?	?
				33-38	-	0	0	0
				?	?	?	?	? died in infancy

Continued...

Table 2 (Con't.) Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased).

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Fisch etal 1969	30	102	1	?	?	0	+	died at 2 mo. CHD, agenesis spleen
			twins	37	-	+	+	hypertelorism, arched palate
				59	-	+	+	arched palate
				33	-	0	+	esotropia
				33	-	0	+	esophageal atresia, anomalous ribs, esotropia
				30	-	0	+	pulmonary stenosis
Frankenburg etal 1968	24-32	54	0	25	-	+	+	C.P. esotropia
				12	-	+	+	pescavus, C.P. EEG abn.
Levy and Shih 1974	>20	56	?	67	-	0	0	0
				61	-	0	0	0
	>20	"dull normal"	?	"slow development"	-	0	0	0
MacCready and Levy 1972, Levy etal 1970	35	"retarded"	0	?	?	?	?	? died in infancy

Continued...

Table 2 (Con't.) Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased).

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
				?	?	?	?	? died infancy
				?	?	?	?	?died infancy
				60	-	?	?	0
				46	-	?	?	0
	28	45	0	79	-	?	?	0
				83	-	?	?	0
	"PKU"	"retarded"	?	retarded	-	?	?	0
Jervis 1937	"PKU"	"imbecile"	?	retarded	+(-)	?	?	?
				retarded	+(-)	?	?	?
				N	-	?	?	?
				N	-	?	?	?
	"PKU"	"retarded"	?	"idiot"	+(-)	?	?	motor disability died at 5 yr.
Goldstein etal 1973	20-23	85	0	N	-	0	0	0
				N	-	?	?	0
Huntley and Stevenson 1969	18-24	76	10	72	-	+	+	CHD

Continued...

Table 2 (Con't.) Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased).

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
				?	-	+	+	died at 16 mo, infection, CHD, CDH, esotropia
				56	-	+	+	esotropia, CDH, EEG
				?	?	+	+	CHD, died at 4 days
				?	-	+	+	CDH, strabismus, CHD (?)
				?	?	+	+	CHD, died at 1 day
Thomas etal 1971	14-22 (+1 theraputic abortion)	67	1	72	-	+	+	seizures
				?	?	+	+	0 died at 6 wks.
Howell and Stevenson 1971				50	-	0	+	0
Scheiben-reiter 1972	16-24	88	0	107	-	0	?	0
				105	+(+)	0	?	0
Hill and Zaleski 1972	PKU	?	?	"Borderline"	+(+)	?	+	0

Continued...

Table 2 (Con't.) Classical phenylketonuria*: Outcome of untreated cases not detected by affected offspring (Unbiased).

*maternal phenylalanine $\geq$ 20 mg/100 ml

Abbreviations:

CDH: congenital dislocation of the hip
CHD: congenital heart disease
C.P.: cerebral palsy
DQ: developmental quotient
EEG: EEG abnormal
IQ: intelligence quotient
IUGR: intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
mo.: month
N: normal
R_x: treatment
wk.: week
vertebrae: vertebrae abnormal
yr.: year
+: present
0: absent
-: none
?: unknown

Table 3 Classical phenylketonuria*: Outcome of untreated pregnancies, ascertainment not known.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU ($\pm R_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Houston etal 1974	"PKU"	55	0	34	-	+	+	0
				32	-	+	+	CHD, hypertonia, gait abn.
				36	-	+	+	hypertonia, gait abn.
				26	-	0	+	hypertonia, gait abn.
	"PKU"	58 (has cleft palate)	1	?	-	+	?	CHD, hypertonia
				?	-	+	?	CHD, hypertonia
				42	-	+	+	CHD, hypertonia, gait abn.
				45	-	+	+	CHD, hypertonia, gait abn.
				?	-	+	+	stillborn
				44	-	+	+	hypertonia, gait abn.
				?	-	?	?	esophageal atresia, cleft palate
	"PKU"	80	1	85	-	0	+	hypertonia, gait abn.
				75	-	+	+	0
				77	+(?)	0	+	0
Hornchen etal 1977	"PKU	?	?	retarded	-	?	+	?
				retarded	-	?	+	?

Continued...

Table 3 (Con't.) Classical phenylketonuria*: Outcome of untreated pregnancies, ascertainment not known.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Jervis 1968 cited by Bickel 1968	30-50	?	?	N	-	?	0	0
				N	-	?	0	0
Jervis 1971 in Howell and Stevensen 1971	30-35	"imbecile"	?	98	-	?	?	0
				N	-	?	?	0
Arthur and Hulme (1970)	21-29	?	?	N	-	?	?	?
Ashley 1969 cited by Hsia 1970	24	retarded	0	retarded	-	?	0	0
				retarded	-	?	0	0
				retarded	-	?	0	0
				retarded	-	?	0	0
				retarded	-	?	0	0
McBean 1969 cited by Hsia 1970	33	?	0	N	-	?	0	0
				N	-	?	0	0
				retarded	-	?	0	0
	26	retarded	0	retarded	-	?	0	+
				retarded	-	?	0	+
				retarded	-	?	0	+

Continued...

Table 3 (Con't.) Classical phenylketonuria*: Outcome of untreated pregnancies, ascertainment not known.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (+R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
				N	-	?	0	0
				N	-	?	0	0
Pitt and Gooch 1974	20	68-85	0	?	?	?	?	? stillborn
			twin	10-35	-	0	+	CHD, seizures
				36-51	-	0	+	C.P., deaf
	25	"dull-normal"	0	36-51	-	0	+	0
				52-67	-	+	?	seizures
				36-51	-	+	+	seizures
				20	-	0	+	seizures, C.P.
				52-67	-	+	+	pigeon chest, C.P., strabismus, seizures
Richards 1964	"PKU"	50	?	43	-	?	?	?
				retarded	-	?	?	?
				retarded	-	?	?	?
Nevsimalova etal 1974	"PKU"	?	?	retarded	-	+	"borderline"	hypotonia
Colombo etal 1967 Colombo 1971	25-43	90	0	<10	+(-)	?	?	EEG
				<10	+(-)	?	?	EEG
				<10	+(-)	?	?	?

Continued...

Table 3 (Con't.) Classical phenylketonuria*: Outcome of untreated pregnancies, ascertainment not known.

* Maternal phenylalanine $\geq$ 20 mg/100 ml

Abreviations:

abn:	abnormal
CHD:	congenital heart disease
C.P.:	cerebral palsy
DQ:	developmental quotient
EEG:	EEG abnormal
IQ:	intelligence quotient
IUGR:	intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
N:	normal
R_x:	treatment
+:	present
0:	absent
-:	none
?:	unknown

offspring of PKU women (Hanson 1970). Although the reporting of cases is not always complete, it is evident from Tables 1, 2, and 3 that very few of the offspring of women with blood Phe levels greater than 20 mg/100 ml are completely free of the stigmata of maternal PKU. Over 90% of the children of women not ascertained in a biased fashion were adversely affected in some way. This figure is not substantially lower than the corresponding rate in the families with biased ascertainment indicating that the high incidence of offspring morbidity associated with maternal PKU represents far more than a reflection of reporting bias (Table 6).

When all cases of maternal PKU are considered (Table 6), nearly 90% of the non-PKU offspring of PKU women are mentally retarded and over one half are microcephalic and suffered intrauterine growth retardation. In addition, 36% of these children had some congenital malformation, including many with serious forms of congenital heart disease. There can be little doubt that the presence of classical PKU in a pregnant woman places the developing fetus at serious risk of congenital malformation, growth retardation, and subsequent mental retardation. Unlike the situation with PKU children, there is no effective postnatal treatment for infants damaged in utero by their mother's hyperphe.

The level of maternal hyperphe is thought to be a factor in determining the likelihood that prenatal damage will occur (Hanson 1970; MacCready and Levy 1972; Levy and

Shih 1974, Perry et al 1973; Trouche et al 1974). The significance of determining the safe level if any, of maternal hyperphe stems from two equally important factors. On the one hand, variant forms of PKU have now been described in which the Phe level is elevated, but less than 20 mg/100 ml (Berry 1976). In addition, the current approach to the treatment of the pregnant hyperphe patient is aimed at reducing blood Phe levels by dietary Phe restriction. In both circumstances the relationship of the degree of maternal hyperphe and the attendant risk to the unborn child is not well defined.

Table 4 illustrates the pregnancy outcome of women with Phe levels greater than or equal to 15 but less than 20 mg/100 ml, while comparable data for women with levels below 15 mg/100 ml are shown in Table 5. When the maternal Phe was between 15 and 19 mg/100 ml over 90% of the offspring were later found to be mentally retarded, approximately the same frequency found in the children of women with classic PKU (Table 6). In addition, the frequency of microcephaly, intrauterine growth retardation, and congenital malformation in the 15 to 19 mg/100 ml group are comparable to those of the PKU group (Table 6). The number of women in the 15 to 19 mg/100 ml group is too small to permit accurate assessment of the role of ascertainment bias. In contrast to maternal hyperphe between 15 and 19 mg/100 ml, women with Phe levels less than 15 mg/100 ml have much lower rates of damaged

Table 4 Atypical phenylketonuria, maternal phenylalanine between 15 and 19 mg/100 ml: Outcome of untreated pregnancies.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (+R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Angeli etal 1974 (B)	18	65-80	1	57	-	+	+	ears
				<45	-	+	+	died 5 yrs., facies, ears, CHD, EEG
				36	-	?	+	toes, fingers, EEG
				37	-	?	+	EEG, toes, fingers, facies
				40	-	?	+	CHD, facies, toes, fingers
				36	-	+	+	EEG, ears, facies, toes, fingers
				25	-	+	+	EEG, ears, facies, toes, fingers
Brown and Waisman 1971 (B)	17	83-94	0	63-77	-	0	+	0
				67-98	-	0	+	0
				61-73	-	0	+	esotropia
				71	-	0	+	situs inversus?, EEG
Onisawa etal 1968 (B)	6-15	110	0	N	-	?	?	0
				9-19	+(-)	+	?	CDH, seizures
				91-110	+(-)	?	0	0

Continued...

Table 4 (Con't.) Atypical phenylketonuria, maternal phenylalanine between 15 and 19 mg/100 ml: Outcome of untreated pregnancies.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU ($\pm R_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Perry etal 1973 (B)	14-17	83	2	25	-	?	+	?
				62	-	?	+	?
				79	-	?	+	?
				59	-	?	+	?
(U)	17	90-95	0	"slow learner"	-	?	+	?
				"slow learner"	-	?	+	?
				"slow learner"	-	?	+	?
(U)	15-16	106	0	"slow learner"	-	?	+	?
				"slow learner"	-	?	+	?
Levy and Shih 1974 (U)	12-15	76	?	retarded	-	0	0	0
				retarded	-	0	0	0
				retarded	-	0	0	0

Abbreviations:
(B): biased, ascertained because of an affected offspring
CHD: congenital heart disease
DQ: developmental quotient
ears: ears abnormal
EEG: EEG abnormal
facies: abnormal facies

Continued...

Table 4 (Con't.) Atypical phenylketonuria, maternal phenylalanine between 15 and 19 mg/100 ml: Outcome of untreated pregnancies.

Abbreviations (con't.):

- fingers: fingers abnormal
- IQ: intelligence quotient
- IUGR: intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
- N: normal
- R_x: treatment
- toes: toes abnormal
- (U): unbiased ascertainment
- yrs.: years
- +: present
- 0: absent
- -: none
- ?: unknown

offspring (Tables 5 and 6). Again the number of cases is small, but it does appear that the risk to the unborn infant is considerably lower in women whose blood Phe level is less than 15 mg/100 ml. More information is required before it can be concluded that women with hyperphe of less than 15 mg/100 ml are not at greater risk of bearing abnormal children than women with normal Phe levels.

Based on available clinical data however, it would be premature to accept less than 15 mg/100 ml as the "safe" level of maternal hyperphe. In the studies reviewed, the maternal Phe level was often not determined during pregnancy, and in fact, was often measured years after the birth of the affected child. A number of different techniques for measuring Phe have been employed and, as Brown (1972) has pointed out, these factors can lead to considerable variation in the estimated Phe level, even for the same patient. The Tables (1 through 5) indicate the variation in maternal Phe levels reported in some patients. In many women, only one Phe measurement was made and this could add additional inaccuracy to the estimation of the Phe level during pregnancy. Until more clinical data on the relationship of the maternal Phe level and offspring risk are available, the question of "safe" levels of maternal hyperphe must remain at best, only tentatively resolved.

A few attempts have been made to prevent in utero damage by treating pregnant hyperphe women with low Phe or

Table 5 Atypical phenylketonuria, maternal phenyalanine less than 15 mg/100 ml: Outcome of untreated pregnancies.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Cohen etal 1969 (B)	6	retarded	0	32	+(-)	?	?	0
				56	+(-)	?	?	?
				66	-	?	?	?
					-	?	?	?
				N	-	?	?	?
DeMenibus etal 1967 (B)	12	48-59	0	54	-	0	+	0
				retarded	+(-)	0	+	0, seizures
				retarded	-	0	+	CHD
Ashley 1969 cited by Hsia 1970 (?)	12	N	?	N	-	0	0	0
				N	-	0	0	0
				retarded	-	0	0	0
Berman 1969 cited by Hsia 1970 (?)	13.6	N	?	?	+(?)	0	0	0
Levy and Shih 1974 (U)	3-12 (5 women	N 11 children)	?	N	- (11 children)	0	0	0

Continued...

Table 5 (Con't.) Atypical phenylketonuria, maternal phenyalanine less than 15 mg/100 ml: Outcome of untreated pregnancies.

AUTHOR	MOTHER			OFFSPRING				
	Phe mg/100 ml	IQ	Abortions	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Lund and Ovnbol 1966 (?)	4-12	N	0	?	PKU(?)	?	?	0
				N	-	0	0	0
Woolf etal 1961 (U)	7.5	102	0	N	-	?	?	0
				N	-	?	?	0
				N	-	?	?	0
				101	-	?	?	0
				95	-	0	0	0
(U)	9.9	83	?	N	-	?	?	0

Abbreviations:
(B): biased, ascertained because of an affected offspring
CHD: congenital heart disease
DQ: developmental quotient
ears: ears abnormal
EEG: EEG abnormal
facies: abnormal facies
fingers: fingers abnormal
IQ: intelligence quotient
IUGR: intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
N: normal
R_x: treatment

Continued...

Table 5 (Con't.) Atypical phenylketonuria, maternal phenyalanine less than 15 mg/100 ml: Outcome of untreated pregnancies.

Abbreviations (con't.):

toes: toes abnormal
(U): unbiased ascertainment
yrs.: years
+: present
0: absent
-: none
?: unknown

Table 6 Maternal hyperphenylalaninemia: Summary of untreated cases.

Maternal Phenylalanine mg/100 ml	Ascer-* tainment	Mothers		Pregnancy		non-PKU offspring**					
		Number	% mentally retarded	Number	% Abortions	Number	% Normal	% mentally retarded	% micro-cephalic	% IUGR	% Malformed
≥ 20 mg %	B	18	50%	93	26%	63	3%	93%	54%	37%	25%
	U	21	75%	81	16%	63	9%	87%	79%	61%	40%
	?	15	80%	51	4%	45	20%	80%	53%	70%	29%
	Total	54	67%	225	17%	171	10%	87%	60%	53%	36%
15-19 mg %	B	4	50%	21	14%	16	6%	88%	100%	50%	82%
	U	3	33%	8	0	8	0	100%	62%	0	0
	Total	7	43%	29	10%	24	4%	92%	87%	36%	64%
< 15 mg %	B	2	100%	7	0	4	25%	75%	100%	0	33%
	U	7	14%	17	0	17	100%	0	0	0	0
	?	3	0	6	0	4	75%	25%	0	0	0
	Total	12	25%	30	0	25	84%	16%	11%	0	4%

*B = biased
U = unbiased
? = ascertainment method unknown

**: percentage calculations exclude offspring for which relevant data were not available.

IUGR = intrauterine growth retardation; < 2500 g at term or <3rd percentile for dates.
mental retardation = IQ ≤ 85

low protein diets. At least 12 cases of such treatment have been reported and in only one instance did the baby escape all the stigmata of maternal PKU (Table 7). Currently only three approaches to the management of maternal hyperphe are available: 1) prevention of pregnancy, 2) theraputic abortion, and 3) dietary Phe restriction during pregnancy. As the data in Table 7 suggest, there remain, unfortunately, a number of unresolved issues to be faced in the dietary treatment of hyperphe during pregnancy. The upper level at which maternal Phe should be maintained has been discussed, and it is doubtful that levels greater than 15 mg/100 ml are safe. The minimum level of Phe must also be considered, since relative Phe deficiency may be a serious risk to the fetus (Brown and Waisman 1971; Bessman et al 1969). In addition, there is virtually no information available on whether or not the "safe" range of maternal Phe changes during pregnancy, thus allowing for the possibility of less stringent dietary restrictions at some times during pregnancy. It is also not known if critical periods exist when the developing infant is particularly vulnerable to maternal hyperphe. It is very likely however, that treatment must begin very early if congenital malformations are going to be prevented, since the vulnerable period of organogenesis occurs before 10 weeks (Moore 1974). In one instance, Phe levels were maintained between 3 and 15 mg/100 ml from 6 to 40 weeks gestation, yet the infant was born with skeletal anomalies

Table 7 Maternal Hyperphenyalaninemia: Treatment during pregnancy

AUTHOR	MOTHER						OFFSPRING				
	Phe mg/100 ml off R_x	Phe mg/100 ml on R_x	Prior Affected Pregnancy #+/total #	IQ	Duration R_x (wks)	Diet: Phe mg/kg/day	IQ/DQ	PKU ($\pm R_x$)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Allan and Brown 1968	27	3-8	4/5	66	18-42	10	108	-	0	0	0
Arthur and Hulme 1970	10-14	2-7	0/0	65	22-38	20-30	98	-	+	+	0
Bovier-Lapierre etal 1974	15-29	3-15	1/1	73	6-40	20	?	-	0	0	died at 2 mo. CHD, hemivertebrae, ↓myelin
Bush and Dukes 1975	16-23	<6	0/0	48-65	10-42	?	?	-	0	0	died at 2 wks. CHD, ↓myelin
	17	?	0/0	71-81	?	?	Abortion 2 wks. after diet instituted.				
Farquhar etal 1971	17	?	1 PKU	65	?	?	Abortion 8 days after diet intstiuted.				
Farquhar etal 1971 Farquhar 1974	24	3-12	?	55	20-40	?	N	-	+	0	0
Forbes etal 1966	20-30	20-30	2/2	96	13-40	low protein	72	-	0	0	0

Continued...

Table 7 (Con't) Maternal Hyperphenyalaninemia: Treatment during pregnancy

AUTHOR	MOTHER						OFFSPRING				
	Phe mg/100 ml → off R_x	on R_x	Prior Affected Pregnancy #+/total #	IQ	Duration R_x (wks)	Diet: Phe mg/kg/day	IQ/DQ	PKU (±R_x)	IUGR +/0/?	Microcephaly +/0/?	Malformations, Comments
Houstan etal 1974	27	?	?	98	last half preg.	?	N	?	?	?	coarctation aorta
Huntley and Stevenson 1969	16-21 diet not tolerated	?	11/11	99	12-16	?	retarded	-	+	+	CHD
Howell and Stevenson 1971	14-22 (diet did not change Phe levels)	14-22	3/3	>67	21-40	?	50	-	0	+	0
Scheiben-reiter 1972	16-24	16-20	0/2	88	26-40	?	70	+(+)	0	+	gut malrotation

Abbreviations:
(B): biased, ascertained because of an affected offspring
CHD: congenital heart disease
DQ: developmental quotient
ears: ears abnormal
EEG: EEG abnormal
facies: abnormal facies

Continued...

Table 7 (Con't.) Maternal Hyperphenyalaninemia: Treatment during pregnancy

Abbreviation (con't.):
- fingers: fingers abnormal
- IQ: intelligence quotient
- IUGR: intrauterine growth retardation (birth weight less than 2500 g or less than 3rd percentile for gestational age)
- N: normal
- R: treatment
- toes: toes abnormal
- (U): unbiased ascertainment
- yrs.: years
- +: present
- 0: absent
- -: none
- ?: unknown

and severe congenital heart disease which proved fatal (Bovier-Lapierre et al 1974). Critical periods for such effects as mental retardation, seizures, and growth retardation are even less well characterized than are those for structural anomalies.

At another level, theraputic advances are hampered by an almost complete lack of understanding of the pathogenetic mechanisms leading to the in-utero damage in maternal hyperphe. A number of parameters, in addition to the elevated Phe levels, could be involved in the pathogenesis of the observed defects. Plausable factors include the effects of Phe metabolites (Glazer and Weber 1971b), deficiency of branched chain amino acids (Berry et al 1977) or Tyr deficiency (Bessman et al 1978). Abnormalities of serotonin and catecholamine metabolism have been reported in PKU (Scriver and Rosenberg 1973) and these factors might also play a role in maternal hyperphe teratogenesis. Both clinical and experimental studies of maternal hyperphe are needed to examine these issues. The experimental approaches to the study of maternal PKU will now be reviewed.

Experimental Studies

Clinical maternal PKU clearly still has many facets in which current knowledge is incomplete. Experimental approaches to this problem have been undertaken with the hope that a fuller understanding of the biological mechanisms

leading to the in utero damage might have significant clinical implications. As yet no non-human animal has been discovered to have genetic PKU, although this remains a promising research avenue (Jones et al 1971). Animal studies have therefore been focused on attempts to experimentally induce the biochemical, behavioral, and morphological effects of maternal PKU.

All animal models of human disease obviously have inherent limitations based on species differences. Nevertheless, criteria can often be generated to maximize the value of a particular model. Experimental studies of maternal PKU ideally should not only duplicate the maternal disease during pregnancy, but also produce some or all of the recognized deleterious effects on offspring development.

Both classical PKU and atypical PKU are characterized by persistently high plasma Phe levels and normal or low Tyr levels. Sustained maternal hyperphe, greater than 15 mg/100 ml, in the absence of significant elevation of Tyr is obviously essential in any experimental study of maternal PKU. Concomitant suppression of maternal hepatic Phe hydroxylase activity, the defective enzyme in PKU, is also important. Excretion of the Phe metabolites found in the urine of PKU patients, particularly phenylpyruvic acid (PPA), should also be sought in the maternal animal. These biochemical manifestations of PKU ideally should be induced in the pregnant animals throughout the duration of pregnancy.

Maternal hyperphe has been found to adversely affect offspring development in a number of ways and all of these need to be considered in experimental models. Congenital malformation and early pregnancy loss should be carefully looked for in experimental studies. Many malformations are thought to arise during the first trimester and therefore studies in which the PKU-like condition is induced only in the latter stages of pregnancy are unlikely to produce malformations. Similarly the relationship between hyperphe and spontaneous abortion, which is also an early event, needs to be studied in the experimental animals. In addition, the newborn offspring should be assessed for intrauterine growth retardation and microcephaly. Offspring should be allowed to mature and then be tested on a battery of behavioral tests in order to assess neurological function. Brains of the offspring should be examined macroscopically and microscopically for evidence of disrupted myelination or other pathology.

In addition to the parameters just outlined, a number of common procedural difficulties must be avoided. It is essential that appropriate control groups be included in all studies. Experimental induction of hyperphe may be accompanied by compromised maternal nutrition. Improved palatability of diets and/or the use of pair-fed controls may be necessary to overcome the potentially confounding effects of inadequate maternal nutrition. In maternal hyperphe the developing offspring are exposed to abnormal Phe metabolism only while

in utero, in contrast to the infant born with PKU who is exposed to hyperphe only following birth. Since the biologic interaction between the abnormal maternal metabolism and the feto-placental unit is unique, induction of experimental hyperphe therefore must be limited to the duration of gestation so that the prenatal and postnatal developmental effects of hyperphe are not confounded. In addition, although the infants of hyperphe women may consume excessive Phe from breast milk (Arthur and Hulme 1970; Fisch et al 1967, 1969; Howell and Stevenson 1971; Yu and O'Halloran 1970; Valdivieso et al 1973), insufficient clinical data are available to assess the significance of this additional Phe intake. It therefore does not seem appropriate to continue experimental maternal hyperphe during lactation unless the study is specifically designed to allow prenatal and postnatal effects of the maternal hyperphe to be clearly distinguished. In addition, immediate neonatal cross-fostering of offspring among the hyperphe and control groups is important to account for variations in maternal rearing behavior which could potentially influence development. These criteria will be considered in the following review of the experimental maternal PKU literature.

Early work involved treating pregnant animals with large doses of Phe while more recent studies have often included the administration of p-chloro-D, L-phenylalanine (pCPA) an inhibitor of Phe hydroxylase.

The first reports of experimental maternal hyperphe involved a small uncontrolled study in which a group of rabbits were injected daily with D, L-Phe throughout gestation as well as prior to pregnancy and during lactation (Ammon 1961; Wassmuth 1958; cited by Karrer and Cahilly 1965). These animals did not excrete significant amounts of PPA. Only 14 offspring were produced, 5 of which died during the neonatal period, and the remainder were said to be "nervous" and "unruly". Further data are awaited from these authors, preferably without the confounding effects of both prepartum and postpartum treatment, so that this model can be adequately evaluated.

Waisman and his colleagues, in the first of a series of reports on their efforts to induce hyperphe in pregnant rats, found that feeding rats a diet containing both 5% D, L-Phe and 5% Tyr resulted in neonatal death (Auerbach et al 1958). Reducing the supplements of each amino acid to 3.75% apparently resulted in normal prenatal development. It is difficult to determine the explanation for the mortality because of the lack of data on maternal food intake, Phe and Tyr levels, fetal weight, and the duration of treatment. Plasma Phe and Tyr levels are reported, (apparantly only for postnatally treated animals) for animals fed either a 5% D, L-Phe or a 5% Tyr diet, but not for animals fed the combined diet, ie the diet associated with offspring death. In addition, the data presented in this report are contradicted by a later publication from the same group (Kerr and Waisman

1967) in which rats fed a 5% D, L-Phe 5% Tyr diet both prior to and during pregnancy carried normal fetuses to term. In the later study, maternal Phe concentrations averaged 11.6 mg/100 ml. Tyr levels were not reported. The conflicting results from the same workers using the same dietary manipulation are difficult to reconcile. The low maternal Phe level, some of which must be composed of the nonphysiologic D-isomer, renders this experimental regimen of questionable potential as a model of maternal PKU.

Waisman's group followed this preliminary report with more systematic study of the effects of feeding Phe supplemented test diets to pregnant rats. Rats were given either 5% Phe, 7% Phe, or 4% D, L-Phe 3% Phe test diets throughout pregnancy and lactation (Boggs et al 1962; Boggs and Waisman 1962). Although the lactating females were unable to satisfactorally nurse their entire litters due to decreased milk production no mention was made of abnormalities in the offspring. Neither data on maternal Phe and Tyr levels nor fetal weight were provided. In a subsequent publication these workers reported maternal and fetal plasma Phe and Tyr levels obtained from pregnant rats fed either a 5% Phe or 7% Phe test diet during the last 10 days of gestation (Boggs and Waisman 1964). Maternal Phe averaged 8.7 and 14.5 mg/100 ml in the 5% Phe and 7% Phe diet groups respectively, while Tyr was elevated to 3.4 and 6.2 mg/100 ml in these animals. Fetal to maternal ratios of plasma Phe

ranged between 2.2 and 2.4 and did not differ between control and test diet animals. Maternal body weight was strikingly decreased by the Phe diets, although fetal weight was not affected. Fetal death or abnormality did not occur. Maternal hepatic Phe hydroxylase activity was reduced to 50% of control in the Phe test diet animals. Kerr and Waisman (1967) provided additional data on the effects of 5% Phe and 7% Phe test diets fed to rats from 12 weeks prior to conception and throughout the subsequent pregnancy. Maternal Phe levels were elevated to only 5.5 mg/100 ml in the 5% Phe group and 8.7 mg/100 ml in the 7% Phe group. Tyr levels were not reported. There was no fetal mortality in these groups and fetal abnormalities were not reported. It is not clear why maternal plasma Phe concentration was lower in the second study, although the duration of test diet treatment may have been a factor. Waisman et al (1964) have briefly reported some follow-up study of rats whose pregnant mothers were fed a 5% Phe diet prior to and throughout pregnancy. These newborn rats were also fed a 2.5% Phe supplemented milk diet from birth until 8 days of age when both light and electron microscopic evidence of retarded myelination was observed in the brains of these animals. The relationship, if any, of these defects to the maternal Phe levels (which are not reported in this paper) however, is subject to question both on the grounds of confounding prenatal and postnatal Phe treatment and the basis of the well known

observation that in the rat, brain myelination is primarily a postnatal event (Dobbing 1973; Ciba 1972). In addition there were no behavioral measures to assess brain function in similarly treated animals.

Kerr and Waisman (1967) reported the effects of feeding pregnant rats test diets supplemented with varying amounts of Phe, D, L-Phe, or combinations of Phe, D, L-Phe and other amino acids. The maternal Phe concentration of 21.0 mg/100 ml found in the 4% Phe 4% D, L-Phe group was the only one to approach levels found clinically in pregnant PKU women. These animals were able to carry normal fetuses to term but were unable to nurse them effectively while being fed the test diet. No data were provided on fetal weight or maternal Tyr levels. It is probable that the maternal animals consuming the 4% Phe 4% D, L-Phe diet also had significant elevation of plasma Tyr. One must also question how much of the 21 mg/100 ml Phe was composed of the non-physiologic D-isomer.

Waisman has not provided any additional data on his efforts to induce a PKU-like state using Phe supplemented diets in pregnant rats. Further interpretation of his work is hampered by the confounding effects of reduced maternal nutrition, combined prenatal and postnatal treatment, and the lack of appropriate offspring follow-up data.

Loo et al (1962) demonstrated transitory deficits in avoidance learning in the offspring of rats fed a 1% D, L-Phe

diet for an unspecified portion of pregnancy as well as during lactation. The offspring were weaned onto the 1% D, L-Phe test diet and, while eating this diet, were shown to have learning deficits which were at least partly reversible when the same animals were later fed the control diet. This rather loosely designed study included only 6 offspring in each group, no cross-fostering, and no data on maternal Phe or Tyr levels. It is of note that at 16 weeks of age the offspring rats did not have significantly elevated plasma Phe levels while consuming the test diet, and it is therefore doubtful that the maternal animals had significant hyperphe.

Leaf et al (1965) have also shown decreased learning ability in the offspring of rats fed a 7% D, L-Phe diet for both the final two days of pregnancy and for the three weeks prior to weaning. Using a cross-over design at the time of weaning, half the offspring from the Phe fed and control fed groups were given either the 7% D,L-Phe or control diets until the time of behavioral testing. The performance of the animals fed Phe during the preweaning period, whether or not they subsequently ate the Phe or the control diets, was significantly poorer than animals not fed Phe prior to weaning, even if these animals had eaten the Phe diet since weaning. This study clearly demonstrates the existence of a critical period for the effects of the 7% D,L-Phe on rat learning behavior, although it is not possible to distinguish the relative contributions of prenatal vs postnatal treatment.

No data were provided on plasma Phe or Tyr, urinary Phe metabolites, or food intake and weight gain, so again it is not possible to assess the relationship of hyperphe and nutritional factors to the learning deficit.

Thompson and Kano (1965) found that the offspring of rats fed a 3% D,L-Phe 3% Tyr diet throughout pregnancy learned a maze more slowly than control rats whose pregnant mothers ate an unsupplemented diet. The maternal diet had no effect on fetal weight, mortality or abnormality. Plasma Phe and Tyr levels were not measured although PPA excretion was significantly increased in the mothers given the test diet. Since the D-isomer of Phe is known to be rapidly metabolized to PPA by D-amino acid oxidase, the presence of excess PPA in the urine of the maternal rats can not be taken as evidence for hyperphe and subsequent increased activity of the alternate pathways of Phe metabolism (Scriver and Rosenberg 1973). Although this was the first report of offspring follow-up not to be confounded by postnatal treatment, its relevance to maternal PKU is limited by a number of problems. Hyperphe was not demonstrated and the lack of cross-fostering leaves the possibility that differences in maternal behavior may have affected later offspring behavior. Most importantly, it is not possible to ascribe the effects to maternal Phe treatment alone, since no 3% Tyr diet control was included.

Perry et al (1965) have also studied the learning

ability of the offspring of rats fed a high Phe diet during pregnancy. When pregnant females were fed a 7% Phe diet during the final 10 days of gestation (normal pregnancy in the rat is about 22 days), the pups were born alive but died within a few hours of birth. No indication of the cause of death of these pups was provided. Reducing the duration of treatment to the last 8 days of pregnancy greatly reduced this neonatal mortality. Maternal Phe levels ranged from 6 to 61 mg/100 ml and maternal urine contained excessive phenylketones. Maternal Tyr was not estimated, however urinary Tyr was greatly elevated suggesting hypertyrosinemia. Newborn body or brain weights were not reported. Although the number of pups tested was small, there was no evidence of decreased learning ability or decreased brain serotonin. The newborn mortality in this study remains unexplained. The relative importance of the maternal hyperphe needs to be weighed against the probable maternal hypertyrosinemia and the uncontrolled maternal nutritional status. There are no other reports of Phe dependant neonatal mortality which suggest there may be a critical period for this effect, and if confounding factors could be excluded, this deserves further study.

Polidora (1967) has reported the most carefully controlled study of the effects of maternal Phe administration on subsequent offspring behavior in rats. Rats were weaned onto either a 5% Phe diet or an unsupplemented control

diet and subsequently mated while eating these diets. So that the effects of maternal treatment during pregnancy and lactation could be differentiated, all offspring were cross-fostered, either to a Phe treated or control dam. All pups were weaned onto the control diet and subsequently tested in a water maze. During pregnancy the maternal plasma Phe levels ranged from 4.1 to 17.9 mg/100 ml. The newborn pups were not weighed, although at weaning offspring exposed to Phe during both pregnancy and lactation weighed significantly less than all other groups. The role of poor maternal food intake cannot be assessed on the basis of the data provided. No fetal death or malformations were noted. Behavioral testing revealed the unexpected finding that animals exposed to Phe during both pregnancy and lactation made significantly fewer errors in the maze than all other groups. Whether behavioral testing on other types of learning tasks might have revealed adverse effects of the prenatal and/or postnatal Phe treatment is not known. No Tyr data were presented, however it is highly probable, based on the results of Boggs and Waisman (1964) using the same test diet, that maternal Tyr levels were at least moderately increased. Although this study was elegantly designed, the results cast doubt on the utility of feeding excess Phe to rats in order to mimic maternal PKU.

In spite of the inability of dietary Phe supplementation in the rat to adequately reproduce either the biochemical or the behavioral hallmarks of maternal PKU, work

utilizing this approach has continued. Luse et al (1970) were the next to report such a study when they fed 3 rats a 7% Phe diet and injected the animals 5 times each week with 1000 mg/kg of additional Phe. The animals were treated in this manner prior to conception and throughout a total of 4 pregnancies. No control animals were reported. One pregnant female died and another cannabilized her entire litter before it could be examined, thus leaving only 2 pregnancies for study of the offspring. Of these, one female died during parturition after delivering 8 dead fetuses and the remaining female was found to be carrying only dead fetuses when sacrificed on gestation day 21. The fetuses available for study were said to be small and a number were found to have cataracts. The photographs of fetuses noted to have cataracts suggest that these fetuses had been dead in utero for some time prior to delivery, and in the absence of histological study, the significance of these lenticular defects is uncertain. A larger adequately controlled study is needed to confirm these data. The amount of hyperphe the maternal rats experienced was not reported, although significant urinary PPA was excreted. Whether the pregnant females had elevated Tyr levels is not reported, nor is the nutritional status of these animals, a potentially significant parameter considering that 2 of 3 females died during the study.

Pasquier et al (1972) fed one pregnant rat a 10% Phe

diet for the last 4 days of gestation and during lactation. The pups were weaned onto the Phe diet which was later increased to 15% Phe. Animals were killed while still eating the supplemented diet and their livers studied by both light and electron microscopy. Another litter was treated in a similar fashion except that a 5% Phe diet was initially used, beginning on the final day of pregnancy. No gross hepatic pathology was observed however decreased glycogen and the absence of fat droplets were noted in the treated rats when examined with the electron microscope. No data on the condition of other organ systems, maternal Phe, Tyr, nutritional intake, or urinary Phe metabolites was given. Liver disease or pathology is not a characteristic finding in either PKU or the offspring of PKU women. The significance of Pasquier et al's findings is difficult to evaluate, especially in view of the small number of animals, the confounding effects of both prenatal and postnatal Phe treatment, and the likelihood of uncontrolled nutritional deficits in the treated animals.

Iijima et al (1975) have also reported on the effects of Phe supplemented diets fed to pregnant rats. The rats were fed a 3.5% Phe diet for the final 10 days of gestation as well as during lactation. No fetal abnormalities or increased mortality were reported. The newborn pups from the Phe-fed females weighed less than control pups, but it is not clear if the difference is statistically significant.

Control rats were not pair-fed and no data are presented on maternal food intake. In addition, no data on maternal Phe, Tyr, or Phe metabolites were provided. The offspring were fed a 7% Phe diet beyond weaning at which time both Phe and Tyr were elevated. This report does not add any new information concerning the effects of prenatal Phe treatment in rats.

Wapnir et al (1977) have presented the most recent data on the effects of feeding pregnant rats Phe supplemented diets. The rats were fed a 7% Phe diet for the last week of gestation and maternal plasma Phe was found to be in the range of 9.9 to 16.5 mg/100 ml. On the day prior to expected delivery, an additional oral dose of Phe (500 mg/kg) was given 1 hour before the animals were killed. At the time of sacrifice, maternal Phe and Tyr were 51.6 and 9.5 mg/100 ml, respectively. Fetal brain Phe was significantly elevated above controls whereas fetal plasma and brain tryptophan were significantly decreased. Injection of the pregnant females with radioactive tryptophan indicated decreased transport of this amino acid into both fetal blood and brain. In addition, fetal brain pyruvate kinase activity was found to be significantly lower in the Phe group. It is important that these biochemical disturbances in fetal brain be correlated with offspring brain function and morphology. In addition it would be of value to determine if the dietary treatment alone, ie. without the Phe bolus 1 hour before

sacrifice, is also associated with these biochemical abnormalities. It is of note that Wong et al (1972) have demonstrated decreased fetal brain tryptophan concentration following a single maternal Phe injection. Further work must include measures to control for decreased maternal food intake in the face of diets supplemented with high levels of Phe. Similar studies using pCPA treatment to prevent elevated Tyr levels might also be useful, as Copenhaver et al (1973) and Berry et al (1975, 1977) have demonstrated decreased brain tryptophan levels in the fetuses of pregnant rats injected with both Phe and pCPA.

Carver et al (1965) have also studied brain amino acids in the fetuses of rats treated with Phe during pregnancy. Rats were given daily Phe injections of 50 mg throughout gestation. This regimen had no effect on maternal plasma Phe levels when estimated 2 hours following the final injection, although Tyr was increased. Fetal brain Phe and Tyr levels were not affected by the maternal treatment although threonine, serine, glutanine, glycine, alanine, valine, methionine, leucine, isoleucine and arginine were significantly decreased while glutamate was increased. Unfortunately, fetal brain tryptophan was not reported. These data are significant because they suggest that fetal brain amino acid imbalance can be produced by maternal Phe treatment, even in the absence of persistent maternal hyperphe. The relationship of the elevated maternal Tyr to the fetal brain amino

acids is not clear, but it does serve to emphasize the need for developing models which minimize Tyr elevations.

Wong and colleagues (Wong and Justice 1972; Wong et al 1972) examined the effect of maternal Phe treatment on fetal brain polyribosomes. Pregnant rats were injected with 1000 mg/kg of Phe on gestation day 20 and killed one hour later, at which time the maternal Phe level was elevated over 200 times control. Maternal Tyr levels were not determined. Fetal brain Phe content was significantly increased while tryptophan was decreased. In addition, fetal brain polyribosomes were found to be disaggregated and in vitro protein synthesis decreased in the Phe treated group. These data, when considered with those of Wapnir et al (1977) and Carver et al (1965), indicate that maternal Phe treatment in the rat can disrupt the fetal brain in a number of ways. The significance of these abnormalities with respect to clinical maternal PKU remains unclear. It is still necessary to demonstrate that these abnormalities have functional or morphologic correlates as well as a relation to maternal hyperphe which is independant from elevated maternal Tyr.

In the studies in which pregnant rats were treated with Phe alone, maternal Tyr was elevated in all instances in which it has been measured (Boggs and Waisman 1964; Carver et al 1965; Wapnir et al 1977; Wapnir and Dierks-Ventling 1971). It is therefore probable that hypertyrosinemia was present in all studies in which pregnant rats have been

treated with Phe alone. As hypertyrosinemia is never found in maternal PKU, its presence must be seriously considered when interpreting the significance of these experimental studies. Although a number of abnormalities associated with maternal PKU have been demonstrated in the offspring of rodents treated with Phe, numerous procedural shortcomings remain unresolved, and no such study has yet provided an adequate experimental model of maternal PKU.

Waisman's group has also studied the effects of feeding four rhesus monkeys Phe supplemented milk diets during a total of 9 pregnancies. The pregnant females were fed from 0.2 to 1.43 g/kg/day of Phe. During pregnancy mean plasma Phe ranged from 14.7 to 39.3 mg/100 ml and at term maternal Phe and Tyr levels ranged from 7.0 to 56.5 mg/100 ml and 2.1 to 10.0 mg/100 ml, respectively (Kerr and Waisman 1966; Kerr et al 1968). Whether or not the pregnant females excreted excess amounts of Phe metabolites was not reported. Umbilical cord plasma Phe and Tyr levels were also increased in the Phe treated group. Half of the newborns (excluding one which died during delivery secondary to a malpresentation) weighed less than 1 standard deviation below the mean, although all had head circumferences within the control range. Poor maternal weight gain was associated with poor dietary intake and this may be relevant to the low newborn weight. No congenital malformations were found. Seven offspring of the hyperphe monkeys were subsequently

studied in a number of behavioral tasks and found to respond differently in a frustration situation and have learning deficits (Kerr et al 1968; Chamove and Davenport 1970; Chamove et al 1973). Although these studies meet many of the criteria previously outlined, the presence of high maternal Tyr and the confounding effects of poor maternal nutrition require further work. The use of the rhesus monkey to study maternal PKU clearly has great potential and it is hoped that additional monkey data will be forthcoming.

When Phe alone is administered to pregnant animals hyperphe is accompanied by hypertyrosinemia. To more closely mimic PKU, a number of workers have treated pregnant rats with pCPA, an irreversible inhibitor of Phe hydroxylase (Koe and Weismann 1966; Lipton et al 1967; Gal and Millard 1971; Guroff 1969), in order to produce hyperphe with only minimal Tyr elevation.

Foote and Tao (1968) were the first to report on the effects of treating pregnant animals with pCPA. Two rats were injected with 300 mg/kg of both pCPA and Phe on gestation day 14 and then injected with 100 mg/kg of both pCPA and Phe every three days until postnatal day 8. Beginning on postnatal day 6 the pups were also injected every 3 days with 100 mg/kg of both pCPA and Phe until killed for analysis of brain fatty acids between 12 and 31 days of age. Controls consisted of one litter which was injected with vehicle only and a second uninjected litter.

No fetal death or malformations were detected. Newborn body weight and brain weight were not reported. Brains of the treated group were found to accumulate oleic acid less rapidly, especially from 16 to 25 days of age. Although lipid abnormalities have been reported in the brain of a retarded offspring of a PKU woman (Menkes and Aberhard, 1969), it is not possible to conclude that the data of Foote and Tao indicate that maternal hyperphe in the rat causes abnormal brain lipid maturation. Further work requires the use of both pCPA and Phe controls, data on maternal Phe and Tyr levels, and restriction of treatment to pregnancy.

Glazer and Weber (1971a) have studied the effects of brief maternal hyperphe on the incorporation of glucose into macromolecules by fetal rat brain. Pregnant rats were given a single injection of 300 mg/kg of pCPA on approximately gestation day 18 and on the following day two additional injections 2 hours apart of 1000 mg/kg of Phe. One hour after the second Phe injection the maternal plasma total Phenylalanines (PA, which includes both Phe and pCPA) averaged 54.5 mg/100 ml in the pCPA plus Phe treated animals compared to 12.1 mg/100 ml and 5.9 mg/100 ml in the pCPA and saline controls, respectively. Although Phe was not measured independantly from pCPA, it does appear that maternal Phe during the three hours after Phe injections reached levels comparable to those found clinically. Neither maternal plasma Tyr or urinary Phe metabolites were measured. Fetal brain slices from the animals

treated with both Phe and pCPA were found to incorporate significantly less glucose into lipids, proteins, DNA, and RNA. The decreased fetal brain synthesis of macromolecules from glucose was prevented by feeding the pCPA plus Phe-treated females excess glucose. The mechanism by which the maternal Phe and pCPA administration affected the glucose incorporation is not entirely clear. The authors have also showed that in vitro glucose incorporation into macromolecules of normal fetal rat brain was inhibited by PPA, albeit at concentrations greater than fifty times that seen in the blood of PKU patients (Partington and Vickery 1974), and that except for lipid synthesis, this inhibition could be reversed by the addition of glucose to the medium. The authors did not measure maternal or fetal tissue PPA concentrations in the in vivo experiments and it is therefore not possible to assess the role of PPA in macromolecular synthesis in pCPA-Phe treated rats. PA content was elevated in the fetal brains of the Phe and pCPA treated females and the authors have also shown that in vitro, glycolysis in normal fetal rat and fetal human brain is inhibited by Phe (Glazer and Weber 1971b; Weber 1969; Weber et al 1970). The work of Glazer and Weber needs to be expanded to determine whether increased dietary carbohydrate might also protect the rat fetus from the effects of sustained maternal hyperphe, not only at the biosynthetic level, but also functionally and morphologically. In addition, it would be useful to

determine if fetal brain biosynthetic function is altered by maternal hyperphe of longer duration.

Andersen (1976) has also injected near-term pregnant rats with Phe (333 mg/kg/day) and pCPA (60 mg/kg/day), beginning on gestation day 18, 19, or 20 and continuing until delivery. Maternal PA levels reached nearly 40 mg/100 ml 3 hours following injection but fell to only about 5 mg/100 ml within 24 hours. Andersen did not measure Phe independantly from pCPA. He cites his earlier work with 5 day old rat pups injected with Phe and pCPA (Andersen et al 1973) as supporting the suggestion that most of the PA assayed in the pregnant females was composed of Phe and not pCPA. The PA levels of the rat pups following Phe and/or pCPA injections however, were considerably different from those of the pregnant females in the current study, so it is uncertain what fraction of the maternal PA measured is actually Phe. In addition, DelValle and Greengard (1976) have measured Phe and shown that sustained hyperphe does not occur when rat pups are injected with the schedule used by Andersen. Andersen (1976) found that compared to saline treated controls, birthweight was significantly decreased in the experimental group. A number of studies have shown that rats treated with pCPA have decreased food intake and poor weight gain (Copenhaver et al 1970, 1973; Moore and Hampton 1974; Wapnir et al 1970). In the absence of both pCPA and Phe treated control groups as well as information on maternal food intake and weight gain,

it is not possible to determine the relationship of the decreased newborn weight to maternal hyperphe.

Andersen (1976) also showed that PA entered fetal brain much more readily than maternal brain and this could mean that fetal brain may be relatively more sensitive to hyperphe than is adult brain tissue.

Copenhaver's group has reported a series of experiments in which rats were injected with various doses of pCPA and Phe during the final one third of pregnancy. Copenhaver first showed that rats injected with 247.8 mg/kg/day of Phe on gestation days 15 through 20 and 299.5 mg/kg/day of pCPA on gestation days 15 and 17 had significantly decreased food intake accompanied by maternal weight loss (Copenhaver et al 1970). Control rats injected only with Phe ate and gained weight at the same rate as the vehicle injected controls. The pCPA controls also had significantly reduced food consumption and lost weight at an even greater rate than the Phe plus pCPA group. In addition, both the pCPA plus Phe experimental group and the pCPA control group had significantly decreased fetal brain and body weight when compared to Phe and vehicle control groups. No fetal death or malformations were detected in any group. The pCPA controls, in contrast to the pCPA plus Phe experimental group, were given daily pCPA injections and therefore received 3 times more pCPA. Maternal blood PA or Phe levels were not reported, but there can be little doubt that the group

receiving both Phe and pCPA had higher Phe levels. Although pCPA appears to play a role in maternal and fetal weight reduction, because of the differences in the two pCPA treatment regimens, it is not possible to adequately assess the role of maternal hyperphe.

Copenhaver et al (1974) reported further data on the effects of a number of Phe plus pCPA treatment regimens on pregnant rats and their offspring. Injections of 495.6 mg/kg/day of Phe on days 15 through 21 and 399.4 mg/kg/day of pCPA on days 15 through 18 were found to elevate maternal plasma PA to a maximum of 34.1 mg/100 ml 2 hours after the Phe injection; plasma PA fell to only 6.5 mg/100 ml within 24 hours. Treatment with lower dosages of Phe and pCPA resulted in lower maternal plasma PA. Phe was not separated from pCPA for these assays. Tyr was not determined in most of these dose response studies, and it is therefore unknown if Tyr levels rose at the times when PA peaked. The wide swings in PA levels following injection of pregnant rats with Phe and pCPA observed by Copenhaver and by Andersen (1976) are probably of greater magnitude and fall to much lower levels than those of pregnant women with PKU.

Treatment of pregnant rats with various doses of Phe and pCPA on gestation days 18 to 21 and 18, respectively, did not appear to adversely affect fetal body or brain weight, although only a single litter was used for each regimen (Copenhaver et al 1974). A longer treatment period however,

injection of 330.4 mg/kg/day of Phe.and 199.7 mg/kg/day of pCPA on days 15 through 21 and 15 and 18, respectively, significantly reduced both fetal body and brain weight. Maternal PA levels reached a maximum of only 14.4 mg/100 ml in similarly treated pregnant rats, and in the absence of pCPA, Phe,. and pair-fed controls, it is not clear that this reduction in fetal weight is related primarily to maternal hyperphe. Fetal death or malformation was not detected in any group, although mothers treated with pCPA were found to kill their pups within 24 hours of birth. This behavior could be completely prevented by giving the pregnant females concomitant treatment with 5-hydroxy-tryptophan, presumably via replacement of depleted brain serotonin stores (Copenhaver et al 1978). These dramatic changes in maternal behavior caused by prenatal treatment highlight the importance of cross-fostering in order to prevent the confounding of prenatal and postnatal influences on subsequent offspring behavior.

Copenhaver has also demonstrated behavioral deficits in the offspring of 3 pregnant rats injected with 165 mg/kg/day of Phe on days 15 to 21 and 165 mg/kg/day of pCPA on days 15 and 18 (Schalock and Copenhaver 1973). Following the injections maternal plasma PA levels reached only 12.6 mg/100 ml and returned to normal levels within 24 hours of injection. As in his other studies, Copenhaver did not determine Phe separately from pCPA nor estimate maternal Tyr levels. The newborn of the pCPA plus Phe injected

mothers weighed less than the controls. The offspring were later found to be less active and less efficient in learning an appetitive task when compared to the offspring of vehicle injected rats. Once again, interpretation of these results is limited both by the absence of pCPA and Phe treated controls, and by the probable confounding effect of decreased maternal nutrition. In addition, none of the pups were cross-fostered in this study.

Copenhaver has also shown that injection of pregnant rats with 330.4 mg/kg/day of Phe on days 18 to 20 plus 399.2 mg/kg of pCPA on day 18 was associated with significant disaggregation of fetal brain polyribosomes (Copenhaver et al 1973). This study included both pCPA and Phe control groups, both of which resulted in moderate but significantly less polyribosome disaggregation than did combined pCPA-Phe treatment. One hour after the final injection maternal PA levels averaged 21.5 mg/100 ml, of which approximately one half was estimated to be pCPA. Maternal Tyr levels were not significantly elevated in any of the treatment groups. Fetal brain PA were increased dramatically in the two pCPA groups and moderately in the Phe treated group, whereas fetal brain tryptophan content was decreased in all three groups but most significantly in the two pCPA groups. Combined treatment with pCPA and Phe resulted in the most marked deviations from the normal with respect to both fetal brain PA, tryptophan, and polyribosome disaggregation. This study is the only

report from Copenhaver that clearly implicates maternal hyperphe, albeit of a short duration, in the pathogenesis of a fetal abnormality. All of Copenhaver's work is limited however, by the use of an injection schedule which resulted in wide fluctuations of maternal PA levels; in some cases PA returned to normal levels prior to the next scheduled injection. Butcher's group has come the closest to meeting the criteria of an acceptable animal model of maternal PKU.

Butcher (1970) reported that the offspring of 3 rats fed a diet supplemented with 3% Phe and 0.12% pCPA on gestation days 10 to 20 learned a maze significantly more poorly than controls. Offspring from rats pair-fed either a 0.12% pCPA or a regular chow diet did not learn as well as offspring of rats fed chow ad libitum, however, they did perform significantly better than the Phe plus pCPA group. These data again emphasize the importance of both pCPA controls and maternal nutrition in studies of experimental maternal hyperphe. Maternal plasma Phe was said to be increased with a Phe to Tyr ratio similar to that found in PKU. In a later publication, pregnant rats fed the same test diet had plasma Phe levels of 13.9 mg/100 ml while Tyr was only 2.3 mg/100 ml (Berry et al 1975). Although the number of subjects is small and no cross-fostering or Phe controls were employed, the data strongly suggest that maternal hyperphe of approximately 14 mg/100 ml during the last half of pregnancy in the rat is associated with a

learning deficit in the offspring.

In a subsequent study (Berry et al 1975) it was shown that when pregnant rats were fed the 3.0% Phe 0.12% pCPA test diet, fetal plasma and amniotic fluid contained markedly elevated Phe levels. pCPA, as well as Phe, was higher in fetal than maternal plasma, suggesting active placental transport of both amino acids. Fetal brain serotonin content was decreased in both the Phe plus pCPA group and the pCPA control group, suggesting that depletion of fetal brain serotonin cannot be the only factor in the offspring learning deficits. Additional studies demonstrated that leucine, isoleucine, valine and tryptophan were lower in fetal brains collected from rats fed the 3.0% Phe 0.12% pCPA diet during the second half of pregnancy (Berry et al 1977). Fetal brain weight was also reduced in the Phe plus pCPA group. When the 3.0% Phe 0.12% pCPA diet was supplemented with additional valine, leucine and isoleucine and then fed to pregnant rats, both fetal brain weight and subsequent maze learning no longer differed from controls. Supplementation of the Phe-pCPA diet with tryptophan however, did not improve either maze learning or fetal brain weight. The leucine, isoleucine, valine supplementation tended to increase the fetal brain isoleucine and valine levels, and although it had no effect on fetal plasma Phe, it significantly lowered fetal brain Phe content suggesting that amino acid transport into fetal brain may be affected. These data also suggest that the

amino acid imbalance in fetal brain involving isoleucine, valine, and Phe could be directly related to both decreased brain weight and impaired brain function in the offspring of hyperphe rats. Whether the protective effect of valine, leucine and isoleucine is related to changes in amino acid transport in developing brain or perhaps alterations in macromolecule synthesis is not known. It would be informative to determine if supplementation with these branched chain amino acids also affects other biochemical abnormalities found in fetal brain following maternal Phe and pCPA administration (Glazer and Weber 1971a; Wong et al 1972; Wong and Justice 1972; Wapnir et al 1977; Carver et al 1965; Copenhaver et al 1973). If further work can substantiate and clarify the role of isoleucine, leucine and valine in preventing offspring damage in maternal hyperphe, then use of these amino acids may have clinical application. It is of interest that Berry has reported that two teenage patients with PKU showed some improvement in behavioral testing in preliminary studies of dietary supplementation with isoleucine, leucine, and valine (Berry et al 1977).

Butcher's dietary model of maternal PKU meets most of the criteria previously outlined. The model, however, needs to be expanded so that the effects of hyperphe induced through the duration of pregnancy can be assessed. In addition, cross-fostering of the newborns would ensure that any subsequent behavioral abnormalities are not due to

differences in maternal rearing behavior. Excretion of excess amounts of Phe metabolites by pregnant hyperphe rats also needs to be demonstrated, as Berry et al (1975) have done for young nonpregnant rats fed the 3.0% Phe 0.12% pCPA diet.

Two additional reports have appeared in which pregnant rats were treated with pCPA for purposes other than to simulate maternal PKU. Moore and Hampton (1974), while studying the effects of pCPA-mediated serotonin depletion on the length of gestation, found that rats treated orally with 316 mg/kg of pCPA on alternate days from gestation days 2 through 21 entirely resorbed many of their litters. Reduction of the pCPA dosage by one half during the first twelve days of gestation greatly reduced this fetal loss, as judged by litter size at term. Severe maternal weight loss occured in both groups and without the use of pair-fed controls it is not possible to assess the role of pCPA in the fetal loss. This is the only report which has associated pCPA with pregnancy loss as well as the only report in which pCPA has been administered during early pregnancy. No data on Phe or Tyr levels were reported and the importance of hyperphe therefore is not known. No malformations were detected in this study.

Lauder and Krebs (1976) have studied the effects of prenatal pCPA injections on the time of origin of neurons which receive serotonin innervation in the adult brain.

Pregnant rats were injected with 300 mg/kg of pCPA-methyl ester on day 8, followed by daily injection of 100 mg/kg/day until one of days 12 through 16. This regimen was not associated with any fetal loss but did result in a 1 to 2 day delay in the time of origin of neurons in a number of brain nuclei. Whether such delays in the time of neurogenesis have functional correlates and/or are related to hyperphe are intriguing questions which must await further study.

Aims of the Present Study

Although considerable progress has been made in experimental approaches to maternal PKU, no entirely satisfactory model has yet appeared. With the notable exception of the work by Waisman's group with monkeys (Waisman 1968; Kerr and Waisman 1966; Kerr et al 1968; Chamove & Davenport 1970; Chamove et al 1973), all other studies of experimental maternal hyperphe have been done with rats or rabbits. The guinea pig has not previously been used in studies of maternal hyperphe, although attempts to induce hyperphe with Phe diets in postnatal animals have been reported (Hsia et al 1963; Hsia et al 1964; Inouye et al 1967). This species however, has several advantages over other common laboratory animals for studies of maternal diseases affecting offspring development. The guinea pig was therefore selected, on the basis of the reasons outlined below, for study of maternal hyperphe. It was hoped to avoid many of the shortcomings of previous work

by systematically determining the conditions necessary to produce hyperphe in these animals and then to determine the suitability of the guinea pig as a model for maternal hyperphe.

Deficient brain myelination has been reported in three offspring of PKU women (Menkes and Aberhard 1969; Bush and Dukes 1975; Bovier-Lapierre et al 1974), and is a common autopsy finding in the brains of PKU patients (Knox 1972; Scriver and Rosenberg 1973). In most common laboratory rodents brain myelination is almost exclusively a postnatal event, whereas in both the guinea pig and man myelination begins in utero (Chase et al 1971; Dobbing and Sands 1970; Dobbing 1973). The guinea pig is therefore an appropriate animal for the study of prenatal insults in which myelination may be disrupted. In addition, since the brain growth spurt in both man and the guinea pig reaches a peak prior to birth rather than postnatally, as it does in the rat, the guinea pig is also well suited to study the effects of maternal disease on fetal brain growth (Chase et al 1971; Dobbing 1974). Since microcephaly is frequently found in the offspring of PKU women and defective myelination may also be common, the guinea pig was selected for a study of experimental hyperphe. The guinea pig has a number of other advantages over the rat for this type of study. Hepatic Phe hydroxylase activity in the guinea pig is intermediate between the rat and man (Voss and Waisman 1966; Berry et al 1972). Guinea pig Phe hydroxylase also has a developmental ontogeny more similar

to man than does the rat (Friedman and Kaufman 1971; Berry et al 1972). In addition the guinea pig has a relatively long gestation of 67 days in which the hormonal control shifts from ovarian to placental, as it does in human pregnancy. This long pregnancy is well suited for studying the effects of a maternal disease which is present for the duration of human pregnancy. In addition, the unresolved question of critical periods for the teratogenic effects of maternal PKU could be tackled with the use of pregnant hyperphe guinea pigs.

The objective of the present investigation was to establish the experimental conditions necessary to induce stable hyperphe in guinea pigs and to then assess the utility of this species in studies of maternal hyperphe. Studies were designed to assess various regimens of Phe and pCPA administration in order to define the paradigm resulting in persistent hyperphe while at the same time, minimizing Tyr elevation. In addition to Phe, pCPA was also administered to inhibit Phe hydroxylase, the defective enzyme in PKU. The appropriate pCPA treatment was determined by relating the pCPA dosage to both the extent of Phe hydroxylase inhibition and the plasma Phe and Tyr concentrations.

Since the guinea pig is dependant on exogenous ascorbic acid, which in turn has been shown to affect Tyr metabolism (LaDue and Zannoni 1961), studies were also undertaken to determine if dietary Phe and pCPA treatment affected

tissue ascorbic acid levels. Since pCPA treatment has been shown to affect food intake in rats, studies were also designed to maximize food intake by improving test diet palatability.

Christensen and Streicher (1948) have reported data that suggests the fetal to maternal plasma ratio for amino acids is about 5:1 in the guinea pig, considerably greater than the ratio of approximately 2:1 for Phe found in man and rats (Cockburn et al 1970; Ghadimi and Pecora 1964; Lines and Waisman 1971; Wapnir and Dierks-Ventling 1971). The existence in the guinea pig of such a large gradient in favor of the fetal plasma would influence the extent of maternal hyperphe required to duplicate the fetal conditions in maternal PKU. Studies were therefore undertaken to determine the fetal to maternal plasma ratios for both Phe and Tyr at various stages of pregnancy in the guinea pig.

Congenital malformations and abortions occur with an increased frequency in maternal PKU. There have been no studies in which both Phe and pCPA have been administered during the first half of pregnancy. Studies of the effects of inducing stable hyperphe during the first part of pregnancy in the guinea pig were therefore performed. Embryonic tissue was studied for evidence of mortality, malformation, and growth retardation. In addition embryonic yolk sac fluid levels of Phe and Tyr were estimated to determine whether

the early embryo is directly exposed to high levels of Phe in the presence of maternal hyperphe and if such exposure was related to abnormal embryonic development.

Materials and Methods: General

Chemicals: Phe, pCPA, Tyr and NADH were purchased from Sigma Chemical Company (St. Louis, Missouri). Ascorbic acid and sucrose were purchased from BDH Chemicals (Toronto, Ontario). Aspartame was obtained from the G. D. Searle Company (Oakville, Ontario and Skokie, Illinois). Dimethyl-tetrahydropteridine (DMPTH) was purchased from Calbiochem (San Diego, California).

Biochemical Methods: Tissues and fluid samples were stored at -25°C prior to assay.

Fluorometric method for Phe: The method used to estimate Phe was a modification of the technique of McCamen and Robins (1962) as described by Hsia and Inouye (1966). Briefly, serum, plasma, and yolk sac fluid were deproteinized in equal volumes of 0.6M TCA and centrifuged at 5000G for 5 minutes. 20 µl of supernatant was then mixed with 300 µl of succinate buffer-ninhydrin-leucylalanine solution (5 volumes of 0.6M succinate buffer, pH 6.88, 2 volumes of 30 mM ninhydrin and 1 volume of 5mM L-leucyl-L-alanine prepared immediately before each assay). The supernatant-ninhydrin-peptide mixture was incubated at 60°C for 2 hours, then cooled and mixed with 2.0 ml of copper reagent (3 volumes of 0.25 M sodium bicarbonate with 0.39 mM potassium sodium tartrate and 2 volumes of 0.6 mM copper sulfate). The relative fluorescence of the Phe-ninhydrin-copper complex at 515 nm

was then determined using an Aminio-Bowman spectrophotofluorometer with an activating wave length of 356 nm. Four-sided quartz cuvettes were used. Standards containing from 1 to 40 mg/100 ml of Phe in 0.6M TCA were run through every assay. This procedure does not distinguish between Phe and pCPA.

Fluorometric method for Tyr: The method used was a modification of the procedure described by Hsia and Inouye (1966). The sample was deproteinized with an equal volume of 0.6M TCA and centrifuged for 5 minutes at 5000G. 20 μl of clear supernatant was mixed with 50 μl of 12 mM α-nitroso-β-naphthol in ethyl alcohol and 100 μl of 0.07 mM sodium nitrite in dilute nitric acid (1 volume of HNO_3 to 4 volumes water). The mixture was then incubated for 20 minutes at 37°C. 1.5 ml of water and 4.0 ml of ethyl acetate were then added and mixed. The lower (aqueous) phase was collected and allowed to stand at room temperature for 1 hour. The relative fluoroscence at 570 nm was then read using an excitation wavelength of 470 nm in the Aminio-Bowman spectrophotofluorometer. Four-sided quartz cuvettes were used. Standard solutions containing from 0.5 to 20 mg/100 ml Tyr in 0.6M TCA were routinely run through all assays.

Spectrophotmetric determination of Tyr: The procedure used (Zannoni 1976) is a modification of the method of Udenfriend and Cooper (1952). Samples were deproteinized

(plasma with an equal volume of 0.6M TCA and in the Phe hydroxylase assay with 0.1 ml of 20% TCA) and centrifuged at 5000G for 5 minutes. 0.5 ml of acidified supernatant was mixed with 0.25 ml 11.55 mM α-nitroso-β-naphthol (50 mg/25 ml in 95% ethyl alcohol), 0.25 ml of 14.49 mM sodium nitrite (25 mg/25 ml in 20% nitric acid) and 0.3 ml of water. The mixture was incubated for 30 minutes at 55°C and then 5.0 ml of ethylenedichloride was added. The absorbance at 450 nm of the tyrosine-nitroso-naphthol complex in the aqueous phase was determined in a Hitachi Perkin-Elmer double beam spectrophotometer. Standard solutions of Tyr were always run with each assay.

Phe hydroxylase assay: The method was slightly modified from that of Zannoni (1976). Fresh or frozen liver was homogenized (10% w/v), with a Brinkman Polytron in ice cold 0.1M phosphate buffer, pH 7.0, containing 0.01% mercaptoethanol. The tissue homogenate was centrifuged at 15,000G for 15 minutes at 4 to 5°C. 0.1 ml of phosphate buffer was then added to 0.4 ml of supernatant followed by the addition of 0.1 ml of freshly prepared cofactor solution (0.7 μM of NADH and 0.35 μM DMPTH in pH 7.0 0.1M phosphate buffer). The reaction was started by the addition of 0.1 ml of 1.4 μM Phe in pH 7.0 0.1M phosphate buffer. The reaction mixture was incubated in a 27°C shaker bath (70-80 oscillations per minute). After 20 minutes of incubation the reaction was stopped by the addition of 0.1 ml of

ice-cold 20% TCA, centrifuged, and the clear supernatant assayed for Tyr by either the spectrophotometric or fluorometric method. Activity of Phe hydroxylase was calculated as the number of μMoles of Tyr produced per hour per g wet weight of tissue. Assays were run in duplicate and additional controls included samples run without the cofactor solution, without Phe, or without the tissue homogenate; volume deficits were replaced by 0.1 M phosphate buffer in these control samples. With the exception of the assays in Series C, Experiment 1, all assays were performed by the author.

Ascorbic Acid Assay: The method used was as reported by Zannoni et al (1974) except the volumes used were increased. Frozen liver was homogenized in ice-cold 5% TCA using a Brinkman Polytron and centrifuged at 15,000G for 15 minutes at 4 to 5°C. 0.1 or 0.2 ml of clear supernatant was then run through the procedure. 20 μl of ortho-phosphoric acid (85%) was added followed by addition of 0.32 ml of freshly prepared aqueous α,α -dipyridyl (0.5%) and 20 μl of aqueous ferric chloride (3%). The solutions were mixed following each addition and then allowed to stand at room temperature for 15 minutes before the absorbance at 525 nm was determined using a Hitachi Perkin-Elmer double beam spectrophotmeter. Occasionally turbidity developed following the addition of ferric chloride and when this occured the mixture was centrifuged for 5 minutes and the clear supernatant read in the spectrophotometer. Freshly prepared standards containing

from 1.5 to 9.0 μg of ascorbic acid in 5% TCA were carried through each assay.

Amino acid analyzer: Samples were run on the Beckman 120 C Amino Acid Analyzer by the Amino Acid Analyzer Service of McMaster University Medical Centre. Plasma was deproteinized with 10% sulfosalicylic acid, mixed, and centrifuged for 10 minutes at 10,000 RPM. Samples were injected into the Analyzer and run with lithium buffers, pH = 2.80 for the first 250 minutes followed by a pH 4.16 buffer for the remainder of the run. The temperature was changed from 36°C to 50°C at 150 minutes and the complete run lasted 580 minutes. Short runs were found to effectively separate Phe, Tyr and pCPA and these runs were usually used. The short run consisted only of the second buffer (pH 4.16), the high temperature (56°C), and lasted for 250 minutes. Calibration runs using known amounts of amino acids, including pCPA, were also performed.

Paper chromatography: Samples were chromatographed by the Biochemical Genetics Laboratory at McMaster University Medical Centre using the method of Scriver et al (1964). The samples (serum, plasma, or yolk sac fluid) were applied directly to Whatman 3 MM filter paper in volumes of 5, 10 or 15 μl. The chromatograms were developed overnight in n-butanol, acetic acid, and water (12:3:5). They were then dried for one hour, stained with ninhydrin-isatin [ninhydrin

0.25% (w/v), isatin 0.1% (w/v), and lutidine 1% (v/v) in acetone], and heated at 75°C for 15 minutes. The amount of Phe and Tyr was estimated by comparison with known standards. All chromatograms were read by people experienced with this technique. When the routine sample of 10 µl was used the limits of detection for Phe and Tyr were 6 mg/100 ml and 4 mg/100 ml, respectively.

Ferric chloride test: Ferric chloride (0.6M) was mixed with an equal volume of urine. The test was considered positive for phenylketones when the mixture turned distinctly green; occassionally the urine-$FeCl_3$ mixture turned brownish-green and such samples were considered as questionably positive.

Animals: All animals used were Hartley strain guinea pigs unless noted otherwise. A number of suppliers were used and this is noted in the relevant experiment. The guinea pigs were housed in wire bottomed cages in an air conditioned room with a 12 hour light-12 hour dark cycle. Upon arrival in the laboratory, animals were numbered by marking the ears or by ear-punching. Commercially prepared pelleted guinea pig chow (Purina, or Teklad Mills) and clean tap water were available ad libitum, except as noted below.

Ascorbic acid supplements: Ascorbic acid supplements were administered to the guinea pigs either by the oral or intaperitoneal (IP) routes. All ascorbic acid solutions were prepared immediately before administration. For IP

injection ascorbic acid was dissolved in distilled water (20 mg/ml) and neutralized to pH 7.0 with NaOH. Injection volumes were 1 ml/300 g (20 mg/300g) or 2 ml/300g (40 mg/300g). For oral administration, ascorbic acid was dissolved (50 mg/ml) in a sterile sucrose solution (4 g/50 ml) and given via a plastic syringe fitted with a 1 to 2 cm length of soft plastic tubing on the barrel. The volume given was 1 ml/300 g body weight (50 mg/300g).

Mating procedure: Only virgin female guinea pigs were used in pregnancy studies. Vaginal membranes were examined daily and a record kept so that it was possible to anticipate estrus. The guinea pig vaginal membrane, which is a delicate membrane covering the vaginal orifice, remains closed during most of the estrus cycle. Just prior to estrus the membrane begins to open and intromission does not occur unless the membrane is wide open. When females were nearly due to be receptive they were placed with potent males and their vaginal membranes examined twice daily. When a wide open vaginal membrane was found in a female caged with a male, the vagina was flushed with 0.9% saline and the fluid examined for the presence of sperm with a phase contrast microscope. The day sperm were detected was designated day 1 of pregnancy (Kalter 1968) and the pregnant female was then isolated. Vaginal membranes of pregnant females were examined daily to detect either the opening of pregnancy (about day 24) or signs of abortion.

Test diet preparation: Purina guinea pig chow was used as the starting material for all test diets produced in the laboratory. The Purina chow was initially ground to a powder using either a hand operated kitchen style meat grinder or a Waring blender. Appropriate amounts (w/w) of Phe and/or pCPA were then added and mixed well. When cabbage or lettuce was also used it was then added and the mixture run through the meat grinder to crush the vegetable. The appropriate weight of sweetener, either sucrose or Aspartame, was dissolved in distilled water (500 ml/kg diet) and the solution thoroughly mixed with the diet mixture. This dampened mixture was then put through the meat grinder to form pellets which were dryed overnight at room temperature. Test diets were usually consumed within one week of preparation. The percentage of Phe in Phe supplemented diets indicates the amount of supplement (w/w) and not the total Phe content. The Purina chow base contains 0.91% (w/w) Phe (Ralston-Purina specifications).

Statistical Analysis: Analysis of variance (ANOVA) with repeated measures was used to test for experimental group or day effects in those studies in which the design involved measuring a parameter on a number of days in the same animals. ANOVA of Phe and Tyr data for example, was done using repeated measures in those experiments in which guinea pigs were fed test diets for three to four weeks and blood was collected from each animal for Phe and Tyr estimation

on a number test diet days. This analysis requires that data be available for each subject on all the days the relevant parameter was measured. In some cases all the data were not available, usually because sufficient plasma could not be collected. When data were missing, either the missing values were estimated by the method of Cochran and Cox (1957) or that subject or study day (for all subjects) was deleted from the analysis. When estimates were made for the missing values the appropriate number of degrees of freedom were subtracted from the error term in the ANOVA (Cochran and Cox 1957).

One-way ANOVA without repeated measures was also performed when appropriate.

Considerable variability in plasma PA, Phe, and Tyr levels was observed when the guinea pigs were fed Phe and pCPA supplemented diets and the variability tended to increase as the absolute levels of PA, Phe, and Tyr increased. In order to stabilize the variance the plasma levels of PA, Phe, and Tyr were routinely transformed to logarithms prior to performing ANOVA.

When significant effects were detected by ANOVA, differences between groups were assessed with the Student-Newman-Keuls multiple range procedure (Winer 1962).

Series B

Introduction

Although injection of pCPA has been found to maximally inhibit rat Phe hydroxylase activity for over 48 hours (Lipton et al 1967; Guroff 1969), guinea pigs injected with pCPA every 48 hours over a 10 day period did not have significant hyperphe 6 hours after receiving Phe injections (Appendix A). Since a number of workers have attempted to produce maternal hyperphe by injecting rats with both pCPA and Phe (Andersen 1976; Copenhaver et al 1970, 1973, 1974; Schalock and Copenhaver 1973; Foote and Tao 1968; Glazer and Weber 1971a) further studies of the effects on the guinea pig of Phe and pCPA injections were undertaken. Guinea pigs were injected with varying regimens of Phe and pCPA and their blood levels of Phe, PA, and Tyr determined during the 12 hours following the final injections. These experiments were designed to determine if injection of guinea pigs with pCPA and Phe could be used to produce sustained hyperphe adequate for model PKU.

Methods

Eight experiments were performed over approximately a 2 month interval using 8 of the guinea pigs included in

Appendix A. Five of the animals were 177 days of age at the initiation of Series B (Experiment 1), and 251 days of age when the final experiment was done. An additional three animals were of an unknown age.

Animals were injected intraperitoneally with varying dosage schedules of Phe, or Phe plus pCPA, and blood was sampled at intervals during the 12 hours following Phe injection. For injection Phe was dissolved in 0.85% saline and pCPA was suspended in 0.85% saline. pCPA was given at a dose of 250 mg/kg/injection (1.25 ml/100 g body weight) in all experiments. Animals were briefly stunned with CO_2 to facilitate the injections.

In Experiments 1, 2, 3, 4, and 5 blood was collected from either the external jugular or cephalic veins under methoxyflurane anesthesia (Appendix A). In Experiments 6, 7, and 8 blood was obtained from the ophthalmic venous plexus by a modification of the method of Riley (1960). The animals were lightly anesthetized with methoxyflurane following CO_2 induction (Hoar, 1969) and a heparinized capillary tube (75 mm by 1.1 to 1.2 mm I.D., Red Tips, Fisherbrand) was inserted between the eyeball and orbital wall so as to disrupt the venous drainage of the eye. Blood flowed freely through the capillary tube and was collected in a test tube. After approximately 0.5 to 1.0 ml of blood was obtained, bleeding was stopped by removal of the capillary tube and the application of pressure on the

closed eye. Neomycin-prednisone (Optisone, Evsco Parmaceuticals, Oceanside, New York) was usually applied to the eye to prevent infection. In Experiments 1 through 6 serum was collected and in Experiments 7 and 8 heparinized plasma was collected.

Blood Phe, PA, and Tyr levels were assayed fluorometrically. Urine was collected at various intervals after treatment in metal trays suspended below each animal's cage and tested immediately with $FeCl_3$.

Table 8 summarizes the Phe and Phe plus pCPA injection schedules used in Experiment 1 through 8.

Experiment 1: Eight (8) guinea pigs were injected at approximately 1000 hours with 200 mg/kg of Phe at a volume of 1 ml/100 g body weight. Each animal was bled once following the Phe injection so that 2 animals were bled at each of 1, 2, 4, and 8 hours after the Phe treatment. Urine was not collected in this experiment.

Experiment 2: Seven (7) guinea pigs were injected at approximately 0900 hours with 312.5 mg/kg of Phe at a volume of 1.25 ml/100 g body weight. Each animal was bled one time following the Phe injection so that blood was collected from 2 animals at each of 1, 2, and 4 hours and from one animal 8 hours after the Phe injection. Urine samples were not collected.

Experiment 3: In this experiment the animals received one pCPA injection approximately 23 hours prior to

Table 8 Series B

Experimental design summary

Experiment Number	Number of Animals	INJECTION SCHEDULE Dose and number of hours pre or post the first Phe injection. pCPA	Phe
1	8		200 mg/kg
2	7		312.5 mg/kg
3	8	250 mg/kg 23 hours pre	312.5 mg/kg
4	8	250 mg/kg 48 hours pre 250 mg/kg 24 hours pre	312.5 mg/kg
5	8	250 mg/kg 24 hours pre 250 mg/kg 12 hours pre	312.5 mg/kg
6	8	250 mg/kg 24 hours pre 250 mg/kg 12 hours pre	312.5 mg/kg 312.5 mg;kg 2 hours post
7	8	250 mg/kg 24 hours pre 250 mg/kg 12 hours pre	437.5 mg/kg 437.5 mg/kg 2 hours post
8	7	250 mg/kg 24 hours pre 250 mg/kg 12 hours pre 250 mg/kg (simultaneous with Phe)	437.5 mg/kg 437.5 mg/kg 2 hours post

to Phe administration. Eight (8) guinea pigs were injected with 250 mg/kg of pCPA at approximately 1030 hours; at about 0900 hours the following morning each animal received an injection of 312.5 mg/kg of Phe (1.25 ml/100 g body weight). Urine was collected for $FeCl_3$ tests 5 hours after the pCPA treatment, just prior to the Phe injections, and at intervals following the Phe injections. Each animal was bled once following the Phe injection so that blood was obtained at each of 1 hour, 2 hours, 4 hours, and 8 hours after the Phe had been given.

Experiment 4: Animals in Experiment 4 were pretreated with pCPA about 48 hours and 24 hours before receiving Phe injections. Eight (8) pigs received 250 mg/kg of pCPA at 0900 hours on the first day and at 1100 hours the following day. At 1130 hours on the third day all animals received injections of 312.5 mg/kg of Phe (1.25 ml/100 g body weight). Each animal was bled once. Blood was collected from 2 animals at each of 1 hour, 2 hours, 4 hours, and 8 hours after the Phe treatment. Urine was collected 6 hours after the first pCPA injection and just prior to both the second pCPA injection and the Phe injection.

Experiment 5: In this experiment the animals received two pretreatments with pCPA during the 24 hours preceding Phe injection. Eight (8) guinea pigs were injected with 250 mg/kg of pCPA 24 hours and 12 hours before receiving a 312.5 mg/kg injection of Phe (1.25 ml/100 g body weight) at approximately 0900 hours. Each animal was bled

one time. Blood was collected from two animals at 1, 2, and 4 hours after the Phe injection and from one animal at each of 5 and 8 hours following the Phe. Urine samples were obtained approximately 6 and 12 hours after the first pCPA injection and one-half hour, 5 hours, and 24 hours following the Phe treatment.

Experiment 6: Lowden's (Clarke and Lowden 1969; Lowden and LaRamee 1969) work indicated that two Phe injections given 2 hours apart resulted in marked and sustained hyperphe in the neonatal rat. In Experiment 6 a similar regimen was evaluated using 8 guinea pigs pretreated with pCPA. The guinea pigs were given 250 mg/kg of pCPA 24 and 12 hours prior to a Phe injection of 312.5 mg/kg (1.25 ml/100 g body weight) at approximately 0915 hours. Two hours after this Phe injection all the animals received a second Phe injection of 312.5 mg/kg. Six animals were bled twice and two animals were bled one time, all by the intraorbital method. Blood was obtained from two animals just prior to the first Phe injection and 1, 4, 6, 8, 10 and 12 hours following this injection. Urine was collected approximately 8 and 12 hours after the first pCPA injection, just prior to the first Phe injection, and at various times following the second Phe injection.

Experiment 7: The design of this experiment was similar to Experiment 6 except that the dosage of Phe was increased. Eight animals were given 250 mg/kg of pCPA 24

and 12 hours before receiving 437.5 mg/kg of Phe (1.75 ml/100 g body weight) at approximately 0915 hours. Two hours later the animals received a second Phe injection of 437.5 mg/kg. Each animal was bled twice. Blood was obtained from 2 guinea pigs prior to the first Phe injection and from 2 guinea pigs at 1, 3, 4, 6, 8, 10, and 12 hours after the first Phe injection. Urine was collected 5 hours after the first pCPA injection, immediately before the first Phe injection, and at various intervals after the Phe injections.

Experiment 8: Experiment 8 was similar in design to Experiment 7 except that an additional injection of pCPA was included at the time of the first Phe injection. Seven guinea pigs were injected with 250 mg/kg of pCPA 24 and 12 hours before receiving injections of 437.5 mg/kg of Phe (1.75 ml/100 g body weight) and 250 mg/kg of pCPA at approximately 0900 hours. Two hours later these guinea pigs received an additional injection of 437.5 mg/kg of Phe. An additional animal received all three injections of pCPA but no Phe treatments. This animal was bled 1 and 4 hours after the final pCPA injection. The animals treated with both pCPA and Phe were bled 1, 3, 4, 6, 8, 10, and 12 hours following the first Phe injection, 2 animals per time interval. Urine was collected 6 hours after the first pCPA injection, and 5 and 12 hours after the first Phe injection.

Results

Blood levels of Phe and Tyr: The normal levels of

serum Phe and Tyr of the 8 animals used in Series B were determined prior to Appendix A, Experiment 1 (Phe 1.70±0.27 mg/100 ml and Tyr 1.23±0.17 mg/100 ml). These baseline levels are illustrated at time B in Figures 1 through 4.

Figure 1 summarizes the results of Experiments 1 through 4. Following intraperitoneal injection of 200 mg/kg Phe (Experiment 1), serum Phe rose to a mean of 4.2 mg/100 ml within one hour and returned to a normal level by 4 hours. Tyr levels were within the normal range throughout the period monitored and were not affected by this dose of Phe. Administration of 312.5 mg/kg of Phe (Experiment 2) resulted in a similar elevation of Phe (5.8 mg/100 ml) at 1 hour, however following this dose, serum Tyr also rose, to a peak of 5.0 mg/100 ml at 1 hour. The level of Phe returned to normal by 4 hours, while the Tyr level fell more slowly. In these experiments animals were not treated with pCPA, and showed no evidence of persistent hyperphe.

Experiments 3, 4, and 5 were designed to assess the effect of pretreatment with pCPA during the 48 hours prior to Phe administration on subsequent serum PA and Tyr levels. When the animals were pretreated with pCPA 24 hours before receiving Phe, serum PA were markedly raised (mean of 15.5 mg/100 ml) and Tyr was also elevated (5.5 mg/100 ml) one hour after Phe treatment (Experiment 3, Figure 1). Both PA and Tyr however, returned to normal levels within 4 hours of the Phe injection. Pretreatment with pCPA 48 and 24 hours

Figure 1. Series B, Experiments 1 to 4. Mean serum phenylalanines (solid lines) and tyrosine (dashed lines) as a function of the number of hours following injection of phenylalanine. Error bars indicate the high or low value when 2 samples were analyzed. Time B illustrates the baseline concentrations determined prior to the experiment.

Figure 2. Series B, Experiment 5. Mean serum phenylalanines (solid)line and tyrosine (dashed line) as a function of the number of hours following phenylalanine injection. Error bars and Time B as in Figure 1.

Figure 1

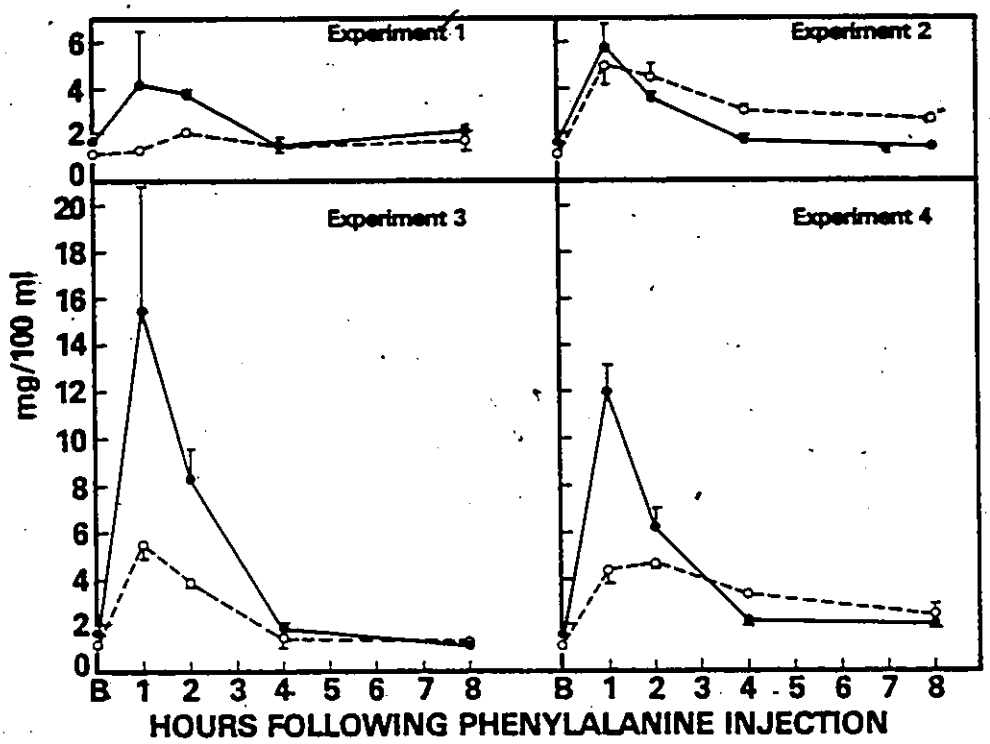
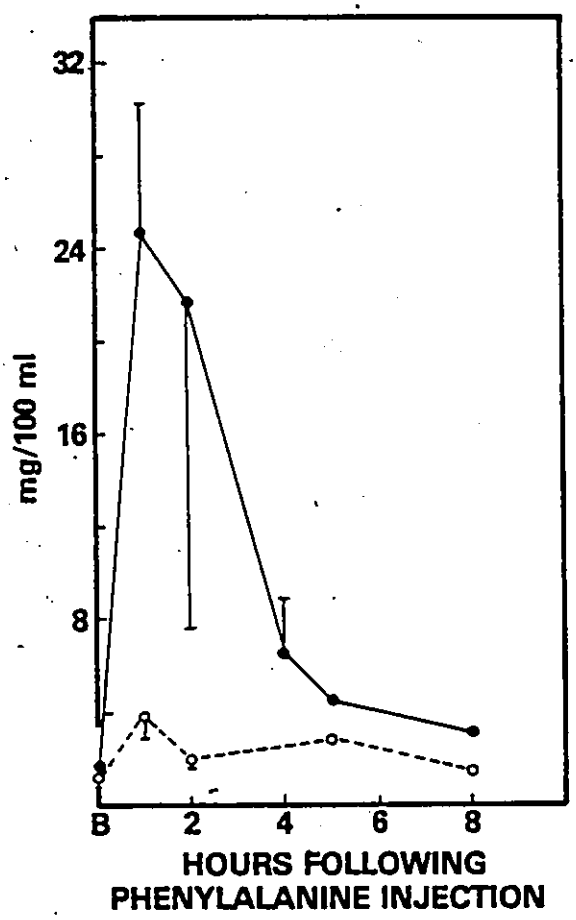


Figure 2



before the Phe injection (Experiment 4) had a similar effect on serum PA and Tyr (Figure 1), although PA reached a peak level of only 12.0 mg/100 ml and Tyr levels returned to normal more slowly than in Experiment 3. The animals in Experiment 5 (Figure 2) were pretreated with pCPA 24 and 12 hours prior to Phe treatment. This regimen produced a greater and more sustained hyperphe than did the previous experiments. PA peaked at 1 hour (24.8 mg/100 ml), declined to 6.6 mg/100 ml at 4 hours, and reached a near normal level of 3.2 mg/100 ml at 8 hours. Tyr was only moderately elevated by this regimen, reaching a maximum of 3.9 mg/100 ml at 1 hour and falling to near normal levels by 2 hours.

Since two pCPA pretreatments during the 24 hours before Phe injection appeared to be more effective than single daily pCPA treatments, this procedure was continued while the frequency and dosage of Phe administration was increased. Experiments 6 and 7 were designed to examine the efficacy of two Phe injections, 2 hours apart, following the two pCPA injections. Figure 3 summarizes the results of Experiments 6 and 7. In each of these experiments blood was also collected from 2 animals just prior to the first of the Phe injections, i.e., approximately 12 hours following the last pCPA injection. The mean PA levels at this time were 3.8 mg/100 ml and 2.6 mg/100 ml in Experiments 6 and 7, respectively with a pooled mean of 3.20±1.54 mg/100 ml. Although the

Figure 3. Series B, Experiment 6 (top panel) and 7 (lower panel). Mean phenylalanines (solid lines) and tyrosine (dashed lines) as a function of the hours following the first phenylalanine injection. Arrows indicate the time of phenylalanine injections. Error bars and Time B as in Figure 1.

Figure 4. Series B, Experiment 8. Mean plasma phenylalanines (solid line) and tyrosine (dashed line) as a function the time following the first phenylalanine injection. Arrows indicate time of phenylalanine injections. Points not on the curves illustrate the total phenylalanines (closed circles) and tyrosine (open circles) levels of an animal which received only pCPA. Error bars and time B as in Figure 1.

Figure 3

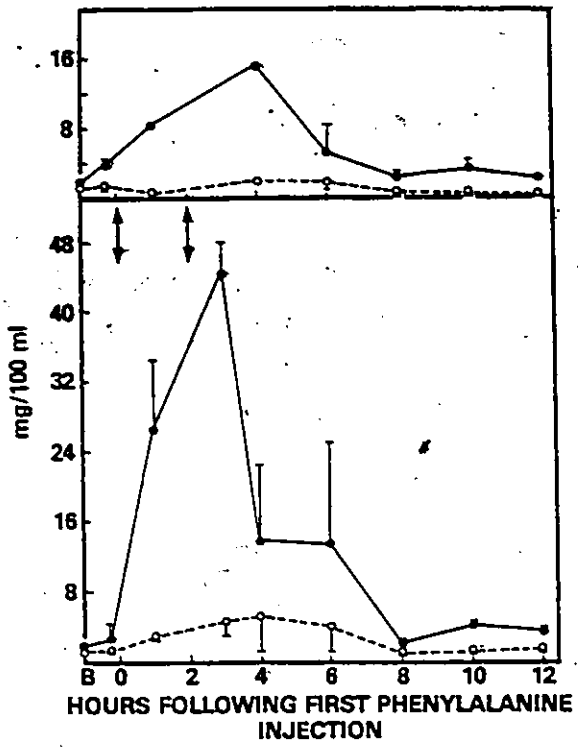
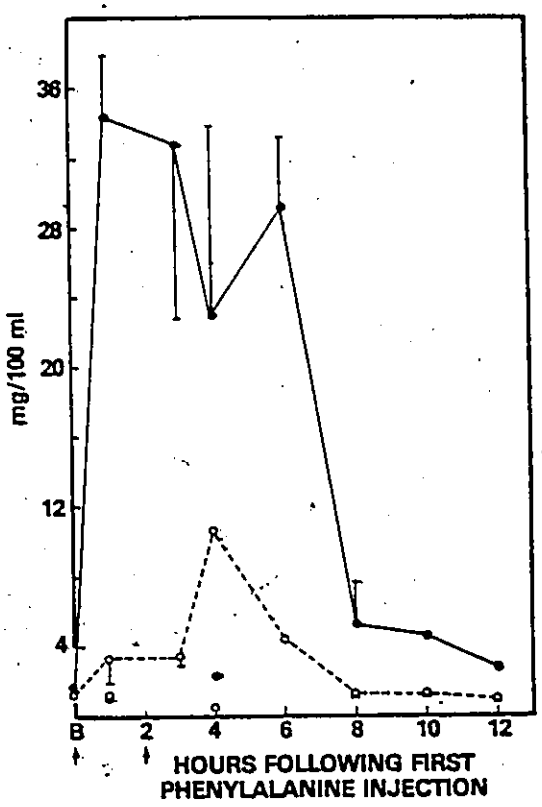


Figure 4



fluorometric assay does not distinguish Phe from pCPA, these data indicate that most of the elevation of PA following Phe injection in Series B must be Phe and not pCPA, since the PA prior to Phe injection are of such a low magnitude compared to the PA levels immediately following the Phe injection.

In Experiment 6 (Figure 3), PA rose to 8.4 mg/100 ml one hour after the first Phe injection and reached a peak of 15.3 mg/100 ml 2 hours after the second Phe injection. Insufficient blood was obtained between one and 4 hours after the first Phe injection to determine serum PA. PA fell to 5.2 mg/100 ml at 6 hours and reached normal levels by 8 hours (2.4 mg/100 ml). Tyr levels was not affected in Experiment 6. In Experiment 7, in which the animals received two Phe injections of 437.5 mg/kg, there was a marked rise in PA one hour after the first injection, mean of 26.6 mg/100 ml, and an even greater peak, 44.6 mg/100 ml, one hour after the second Phe injection. PA levels declined to 13.4 mg/100 ml 6 hours after the first injection and fell to normal levels by 8 hours. Tyr levels rose following the Phe treatments and reached a peak of 5.0 mg/100 ml 4 hours after the first injection. It is noteworthy that one of the 4-hour blood samples had a Tyr level of 9.1 mg/100 ml, an unacceptable Tyr elevation. Tyr levels also fell to normal within 8 hours.

Experiment 8, which was similar to Experiment 7 except that an additional treatment of pCPA was included,

was undertaken to determine the effect of continuing pCPA treatment up to the time of the first of two Phe injections. This experiment is illustrated in Figure 4. One animal received all three pCPA injections but none of the Phe injections. Blood was obtained from this animal one and four hours after the last pCPA injection, and the PA and Tyr levels are indicated in Figure 4 by the points not on the PA and Tyr curves. It is clear that in this animal, one and four hours after the last of 3 pCPA injections plasma PA and Tyr were near or within the normal range. These data, when considered with the PA levels prior to the Phe injections in Experiment 6 and 7 (Figure 3), indicate that the marked elevations of PA observed following Phe injection in Experiments 3 through 8 are primarily a reflection of increased Phe and not pCPA blood levels.

Plasma PA in animals receiving both Phe injections reached 34.4 mg/100 ml at one hour and remained elevated (29.2 mg/100 ml) until 6 hours after the first Phe injection (Figure 4). By 8 hours however, the PA levels had fallen to only 5.2 mg/100 ml and reached near normal levels (2.8 mg/100 ml) by 12 hours. Tyr levels rose moderately, to 3.4/mg/100 ml, 1 and 3 hours after the first Phe injection; however at 4 hours (2 hours after the second Phe injection) the Tyr level was 10.6 mg/100 ml. Tyr levels fell to normal by 8 hours.

Urine $FeCl_3$ Tests: In Experiments 3 through 8 urine for $FeCl_3$ tests was collected at various intervals following pCPA and Phe treatment. The urinary $FeCl_3$ data were analyzed in an effort to determine the relative roles of Phe and pCPA injections in contributing to positive tests. These data are included in Table 9 and are presented as the number of positive tests per total number of tests during the intervals following either pCPA or Phe injection. In the 6 hours following an injection of pCPA, 93% of tests were positive while only 44% were positive during the 6 hours following Phe injection; this difference is highly significant (χ^2 = 15.53; df = 1, $p < .0001$). During the interval from 6 to 12 hours after injection, 62% of tests following pCPA and only 27% of tests following Phe were positive, also a statistically a significant difference (χ^2 = 5.50, df = 1, $p < 0.02$). The data are limited, but at intervals beyond 12 hours, there does not appear to be a difference between animals receiving pCPA and those receiving Phe (χ^2 = 0.06, df = 1). Direct addition of pCPA to fresh urine did not lead to a positive $FeCl_3$ test. Although there was a significant association between pCPA treatment and positive urine tests, a number of positive results were found in animals given Phe. It should be noted that the 3 animals with positive tests within 6 hours of Phe treatment in Experiment 7 (Table 9), had negative urine just prior to this Phe injection. The green colour obtained in positive tests faded within 2 to 3 minutes, in contrast to the persistance of positive reactions in the urine of PKU patients.

Table 9 Series B Number of positive $FeCl_3$ tests/total number of tests. Relation to the time interval since pCPA or Phe injection.

Experiment Number	Hours Following Injection						
	Following pCPA			Following Phe			
	0-6	7-12	13-24	0-6	7-12	13-24	>24
3	5/5		0/7	0/5	0/2		0/6
4	8/8		0/12				
5		13/15	5/8	2/5		2/8	
6		9/15	2/6	6/12	2/5		
7	6/8	0/6		3/3*	2/8		
8	5/5 3/3**	4/6**					
Total	27/29	26/42	7/33	11/25	4/15	2/8	0/6
Total Percentage Positive	93.1%	61.9%	21.2%	44.0%	26.7%	25.0%	0%

* these animals had negative tests just prior to receiving Phe

** these samples taken following combined Phe + pCPA injection.

Series C, Experiment 1

Introduction

The experiments described in Series B were designed to accurately characterize the guinea pig's blood Phe and Tyr response to injections of Phe and pCPA. Even in Experiment 8, in which animals were treated with pCPA three times during a 24 hour period, given Phe concurrently with the final pCPA treatment, and Phe again 2 hours later, plasma PA levels returned to the normal range within 12 hours of the first Phe injection. In addition to the transient hyperphe, plasma Tyr was found to be elevated above 10 mg/100 ml. Previous experiments in Series B had demonstrated that less frequent administration of pCPA resulted in a more rapid fall in blood PA.

It is apparant that in order to produce stable hyperphe in the guinea pig more frequent administration of pCPA and Phe would be required. Because of the large doses and injection volumes already tested in Series B, a more frequent injection schedule was considered to be too traumatic to be compatable with the maintenance of pregnancy; in addition it was also considered logistically impractical to attempt to inject animals more frequently than every 12 hours throughout the duration of a 67 day gestation. It therefore

seemed appropriate to evaluate the efficacy of dietary administration of pCPA and Phe in guinea pigs.

A number of investigators have utilized diets supplemented with Phe and/or pCPA to produce stable hyperphe in rats (eg: Butcher 1970; Berry et al 1975; Berry et al 1977, Wapnir et al 1977; Yuwiller and Geller 1970). Hsia (Hsia et al 1963; Hsia et al 1964; Inouye et al 1967) has reported studies in which Phe alone was added to the diet of guinea pigs, hyperphe was produced, but Tyr levels were also substantially elevated in the study in which they were reported. Given these data, and the observation that guinea pigs eat approximately every 20 minutes throughout the day and night with a maximum between-meal interval rarely exceeding 90 minutes (Hirsch 1973), the addition of Phe and the Phe hydroxylase inhibitor pCPA to guinea pig feed might be expected to produce stable hyperphe with only slight elevation of Tyr.

Preliminary studies in this laboratory have suggested that guinea pigs fed diets supplemented with approximately 1% pCPA and greater than 2% Phe have stable hyperphe and near normal Tyr levels.

The experiments in Series C were undertaken to further evaluate dietary administration of Phe and pCPA and to establish the dietary supplements of these amino acids required to produce stable hyperphe in non-pregnant adult female guinea pigs. In Experiment 1 the optimum dietary

pCPA supplement was determined by monitoring plasma PA, Tyr and hepatic Phe hydroxylase in animals fed diets supplemented with varying amounts of pCPA.

Methods

Twenty-two (22) guinea pigs were assigned to the 6 test diet and Purina chow control groups as summarized in Table 10. All diets, with the exception of Purina chow, were supplemented with 7% sucrose (w/w) and 3% fresh cabbage (w/w) to improve palatability. The following 7 diet groups were included (Table 10):

Group (a): 3.5% Phe test diet, 3 animals.

Group (b): 3.5% Phe 0.75% pCPA test diet, 2 animals.

Group (c): 3.5% Phe 1.0% pCPA test diet, 5 animals.

Group (d): 3.5% Phe 1.25% pCPA test diet, 5 animals.

Group (e): 3.5% Phe 1.50% pCPA test diet, 5 animals.

Group (f): 1.0% pCPA test diet, 1 animal.

Group (g): Purina chow control, 2 animals.

One animal originally assigned to group (b). refused to eat this diet and was therefore fed Purina chow until day 17 of the study when it was given 1.0% pCPA, forming group (f) (Table 10). In order to replace this animal in group (b), one animal was switched from group (a) to group (b) 7 days after the experiment began (Table 10). With the exception of these two animals, the guinea pigs in the test diet groups (a) through (e) consumed the

Table 10 Series C, Experiment 1

Summary of diet groups and animals.

DIET GROUP	GUINEA PIGS		
	NUMBER*	AGE (NUMBER)	SOURCE
3.5% Phe (a)	3 ⟶ 2 (day 7)	112 days	Sunrise Mousery**
3.5% Phe 0.75% pCPA (b)	1 ⟶ 2 (day 7)	112 days	Sunrise Mousery
3.5% Phe 1.0% pCPA (c)	5	112 days (3) 75 days (2)	Sunrise Mousery
3.5% Phe 1.25% pCPA (d)	5	112 days	Sunrise Mousery
3.5% Phe 1.50% pCPA (e)	5	112 days	Sunrise Mousery
1.0% pCPA (f)	⟶1 (day 17)	112 days	Sunrise Mousery
Purina Control (g)	2	96 days	Canadian Breeding Laboratories***

* ⟶ indicates animal placed in or removed from group. The day of the experiment when this change was made is shown in brackets.
** White House Station, N.J.
*** Montreal, P.Q.

supplemented diets for 25 consecutive days.

All animals except the Purina controls received daily oral ascorbic acid (50 mg/300 g). Daily weight and food intake were recorded when the animals were fed supplemented test diets.

Plasma was collected for fluorometric and paper chromatographic estimation of Phe, PA, and Tyr prior to the test diet period, and 8, 11, 17, and 23 days after the test diets were given. The blood samples were obtained by the intraorbital method between 0925 and 1240 hours. The Purina control animals were bled once on day 23 of the study.

Twenty-five (25) days after the study began, all animals were killed by decapitation, the livers quickly removed and frozen on solid CO_2. The liver tissue was kept frozen for 5 days and then packed on frozen CO_2 and sent by air express to Dr. V. G. Zannoni, Department of Pharmacology, New York University, for assay of Phe hydroxylase activity. All tissues remained frozen until assayed; and all samples were processed on the same day (Zannoni 1974, personal communication). Hepatic protein content was measured by the method of Lowry et al (1951).

Results

Phenyalanine Hydroxylase. The Phe hydroxylase activity measured in liver tissue is shown in Table 11.

In the presence of synthetic cofactor, liver from animals consuming diets containing 1% pCPA (with or without Phe) had the lowest detectable enzyme activities. The Phe hydroxylase activities in the 3.5% Phe 1.0% pCPA diet group and from the animal fed the 1% pCPA test diet were 25% and 30% of Purina control activity, respectively. The mean activities of all other experimental groups, while showing some inhibition, were greater than in the 1.0% pCPA animals, indicating that within the range of pCPA supplements studied, inhibition was maximal at the 1.0% pCPA level. The inhibition of hepatic Phe hydroxylase by diets containing 1% pCPA has been confirmed in this laboratory and is discussed below (Series C, Experiment 5).

When synthetic cofactor was not added to the incubation, tissues from the 3.5% Phe 1.0% pCPA group had a mean activity of only 1.2% control, the lowest among all groups. Surprisingly, the animal fed the 1.0% pCPA diet had more activity (42% control) in the absence than in the presence of the synthetic cofactor (30% control). In the absence of synthetic cofactor, all other animals consuming diets containing at least 1.0% pCPA, showed considerably more inhibition than in the presence of exogeneous cofactor. The protein content of the liver homogenates were similar in all groups (Table 11).

Table 11 Series C, Experiment 1 Effect of dietary p-chlorophenylalanine supplement on hepatic phenylalanine hydroxylase activity.

DIET GROUP	BODY WEIGHT (g) N**	Mean ±S.D.	LIVER WEIGHT (g) N	Mean ±S.D.	LIVER PROTEIN mg/ml homogenate (% Purina control) Datum	Mean	PHENYLALANINE HYDROXYLASE μMoles Tyr/hour/g wet weight (% Purina Control) +DMPTH* Datum	Mean	-DMPTH Datum	Mean
Purina Chow (g)	2	749.5±181.7	2	33.8±2.4	17.50 14.56	16.03 (100%)	14.70 6.03	10.37 (100%)	12.69 5.04	8.82 (100%)
3.5% Phe (a)	2	503.0±35.4	2	17.6±0.8	17.12 15.62	16.37 (102%)	10.13 5.28	7.70 (74%)	9.42 3.09	6.26 (71%)
3.5% Phe 0.75% pCPA (b)	2	488.0±26.9	2	19.5±0.7	17.50 15.12	16.31 (102%)	3.48 4.41	3.94 (38%)	2.40 3.87	3.14 (36%)
3.5% Phe 1.0% pCPA (c)	4	477.5±31.5	4	20.05±2.54	17.62 17.50	17.56 (110%)	2.10 3.09	2.60 (25%)	0 0.21	0.10 (1.2%)
3.5% Phe 1.25% pCPA (d)	4	401.2±22.5	4	14.95±1.61	15.88 17.94	16.91 (105%)	7.20 6.36	6.78 (65%)	1.26 1.17	1.22 (14%)
3.5% Phe 1.50% pCPA (e)	4	386.8±9.1	4	15.20±1.68	18.12 15.94	17.03 (106%)	3.57 5.76	4.67 (45%)	0.15 0.84	0.50 (5.6%)
1.0% pCPA (f)	1	443	1	19.0	16.25	(101%)	3.12	(30%)	3.73	(42%)

* Dimethytetrahydropteridine

** N = number of animals

Plasma PA, Phe and Tyr: Plasma levels of Phe, PA and Tyr were measured fluorometrically on test diet days 8, 11, 17 and 23. Figure 5 illustrates the mean levels on each of these days plus the mean during the entire test diet period for groups (a) through (e). In addition to these data, Table 12 also includes the data from the animals fed the 1% pCPA, group (f), and Purina chow diets, group (g), as well as the Phe:Tyr and PA:Tyr (P/T) ratios for all groups.

Compared to prediet levels (Figure 5), all diets containing both 3.5% Phe and pCPA were associated with sustained elevation of plasma PA with only a moderate rise in Tyr levels throughout the test diet period. Animals fed the 3.5% Phe diet, group (a), however, had comparable increases of both Phe and Tyr, with mean levels during the test diet period of 7.00 and 7.68 mg/100 ml, respectively (Figure 5, Table 12). This concomitant elevation of both Phe and Tyr is reflected by the Phe:Tyr ratio in group (a), which did not rise significantly above unity, in contrast to the P/T ratios in other test diet groups (Table 12).

The plasma PA data from the test diet period in groups (a) through (e) were analyzed by ANOVA with repeated measures. The data were transformed to logarithms in order to stabilize the variance which was found to increase as

Figure 5. Series C, Experiment 1. Mean plasma phenylalanines and tyrosine as measured fluorometrically. Error bars indicate standard deviation.

Figure 5

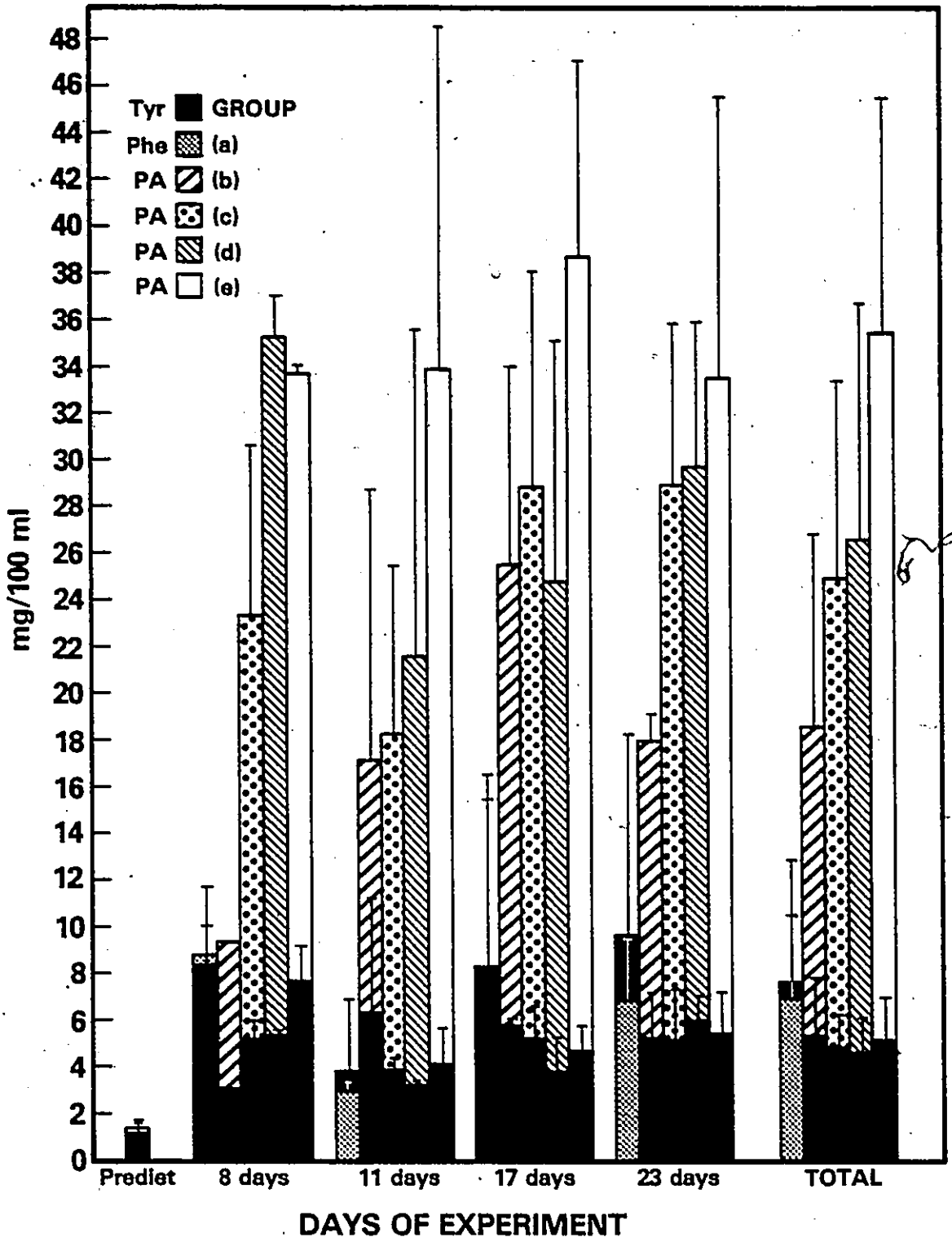

48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
mg/100 ml
Tyr GROUP
Phe (a)
PA (b)
PA (c)
PA (d)
PA (e)
Predlet
8 days
11 days
17 days
23 days
TOTAL
DAYS OF EXPERIMENT

Table 12 Series C, Experiment 1 Mean (±S.D.) plasma phenylalanine (Phe), total phenylalanines (PA) and tyrosine (Tyr) in mg/100 ml as measured fluorometrically.

DIET GROUP		DURATION OF EXPERIMENT 8 DAYS	11 DAYS	17 DAYS	23 DAYS	TOTAL
3.5% Phe (a)	N*	3	2	2	2	9
	Phe	8.80 ±1.24	3.00 ±0.28	8.40 ±7.07	6.90 ±2.69	7.00 ±3.64
	Tyr	8.40 ±3.39	3.85 ±3.18	8.40 ±8.34	9.70 ±8.63	7.68 ±5.21
	**P/T	1.05	0.78	1.00	0.71	0.91
3.5% Phe 0.75% pCPA (b)	N	1	2	2	2	7
	PA	9.4	17.20 ±11.60	25.60 ±8.48	18.10 ±1.13	18.74 ±8.11
	Tyr	3.1	6.30 ±4.95	5.80 ±0.28	5.30 ±1.98	5.41 ±2.44
	P/T	3.03	2.73	4.41	3.42	3.46
3.5% Phe 1.0% pCPA (c)	N	4	5	5	5	19
	PA	23.42 ±7.31	18.32 ±7.16	28.98 ±9.20	28.96 ±6.92	25.00 ±8.45
	Tyr	5.22 ±0.79	3.92 ±0.52	5.20 ±1.43	5.24 ±2.13	4.88 ±1.40
	P/T	4.49	4.67	5.57	5.53	5.12
3.5% Phe 1.25% pCPA (d)	N	2	5+	5	5	17+
	PA	35.30 ±1.84	21.74 ±13.85	24.84 ±10.35	29.80 ±6.16	26.62 ±10.27
	Tyr	5.45 ±0.07	3.23 ±0.21	3.92 ±1.34	6.00 ±1.11	4.68 ±1.50
	P/T	6.48	6.73	6.34	4.97	5.69

Continued...

Table 12 Series C, Experiment 1 (Con't)

DIET GROUP		DURATION OF EXPERIMENT 8 DAYS	11 DAYS	17 DAYS	23 DAYS	TOTAL
3.5% Phe 1.50% pCPA (e)	N	2	4	5++	4	15++
	PA	33.75 ±4.31	33.98 ±14.65	38.78 ±8.37	33.63 ±12.07	35.45 ±10.22
	Tyr	7.70 ±1.56	4.08 ±1.64	4.72 ±1.11	5.45 ±1.82	5.17 ±1.81
	P/T	4.38	8.33	8.22	6.17	6.86
1.0% pCPA (f)	N				1	
	PA				16.2	
	Tyr				1.8	
	P/T				9.00	
Purina Control (g)	N				2	
	PA				1.95 ±0.49	
	Tyr				1.25 ±0.07	
	P/T				1.56	

* number of samples analyzed
** Phenylalanine: tyrosine or total phenylalanines: tyrosine ratio.
\+ Tyrosine not measured in two samples.
++ Tyrosine not measured in one sample.

the mean PA level increased. This ANOVA revealed a significant effect for the number of days on the test diets ($F = 6.632$, $df = 2, 26$ $p < .005$). Comparison of the data for each day of blood sampling by the Student-Neuman-Keuls multiple range procedure indicated this effect for days was due primarily to the lower PA levels on day 11. The mean PA level on day 11 was significantly ($p < .05$) less than the PA levels on days 17 and 23. Day 8 was excluded from this repeated measures ANOVA because of the number of missing samples (Table 12), although 2-way ANOVA without repeated measures which included day 8 revealed similar results.

As the dietary supplement of pCPA increased from 0.75% to 1.50%, the mean PA levels also increased. ANOVA also revealed that the composition of the test diet significantly affected the plasma PA levels ($F = 12.406$, $df = 4, 13$ $p < .001$). Multiple comparisons using the Student-Neuman-Keuls procedure indicated that plasma PA levels of the 3.5% Phe diet group (a) were significantly ($p < .05$) lower than all groups which in turn did not differ significantly from one another. The fluorometric assay used for Phe and PA measurement does not distinguish pCPA from Phe. Both PA [Phe in group (a)] and Phe were measured in a number of plasma samples by fluorometry and paper chromatography, respectively. These data are illustrated in Table 13. Due to the limited number of samples which could be run through both procedures, the data from each diet group have been

Table 13 Series C, Experiment 1 Comparison of fluorometric and paper chromatographic measurement of plasma phenylalanine, total phenylalanines, and tyrosine, mean ±S.D.

Diet Group	Number of Samples	Phenylalanine(s) (mg/100 ml) Fluorometic	Phenylalanine(s) (mg/100 ml) Paper +(% of fluorometric)	Tyrosine (mg/100 ml) Fluorometric	Tyrosine (mg/100 ml) Paper +(% of fluorometric)
3.5% Phe (a)	3	7.07 ±1.92	6.33±2.89 (89.5%)	8.53 ±6.43	5.83±5.39 (68.3%)
3.5% Phe 0.75%pCPA (b)	3	11.83 ±4.74	8.00±6.00 (67.6%)	4.47 ±2.01	3.33±2.08 (74.5%)
3.5% Phe 1.0% pCPA (c)	11*	25.35 ±7.97	16.00±7.84 (63.1%)	4.68 ±1.53	5.50±2.48 (117.5%)
3.5% Phe 1.25%pCPA (d)	12**	28.83 ±9.33	13.54±6.75 (47.0%)	5.12 ±1.39	4.38±1.45 (85.5%)
3.5% Phe 1.50%pCPA (e)	7**	32.79 ±13.06	17.93±10.93 (54.7%)	5.24 ±1.50	4.79±2.12 (91.4%)

* Includes 2 samples taken on day 25 of experiment

** Includes 1 sample taken on day 25 of experiment

\+ For purposes of calculation paper chromatographic Phe and Tyr below the level of detection, 6 mg/100 ml for Phe and 4 mg/100 ml for Tyr, were assumed to be 3 mg/100 ml and 2 mg/100 ml respectively.

pooled across the entire test diet period. Unlike the plasma PA levels, the paper chromatographic Phe estimations did not rise as the dietary pCPA supplement increased above 1.0% (Table 13). This is supported by the one-way ANOVA of the pooled paper chromatographic Phe data which did not reveal a significant effect for the test diet group ($F = 1.855$, $df = 4, 14$).

The samples from the 3.5% Phe 1.0% pCPA group averaged nearly 16 mg/100 ml of Phe, 62% of the fluorometric PA level; Phe constituted a smaller fraction of the PA in the groups with dietary pCPA supplements greater than 1.0% (Table 13).

Fluorometric and paper chromatographic estimates of plasma Phe agreed well in the 3.5% Phe group, as did Tyr estimates in all groups (Table 13).

The animal consuming the 1.0% pCPA diet had a PA level of 16.2 mg/100 ml and a normal Tyr level on day 23 (Table 12); paper chromatography of this sample indicated that the Phe level was less then 3 mg/100 ml. The effects of the 1.0% pCPA diet on plasma PA levels have been examined in more detail and are included in Series C, Experiment 2.

Plasma Tyr levels were moderately elevated above the pretest diet levels in groups (a) through (e) (Figure 5, Table 12). The fluorometric plasma Tyr data from the test diet period were also transformed to logarithms and subjected to ANOVA with repeated measures. In contrast to the PA data

however, ANOVA of the Tyr data did not reveal significant effects for either the test diet day ($F = 0.423$, $df = 2, 24$) or the test diet group ($F = 0.239$, $df = 4, 13$).

The plasma PA, Phe, and Tyr data indicate that feeding guinea pigs test diets supplemented with both Phe and pCPA resulted in sustained elevations of plasma PA and Phe with only moderate elevation of Tyr. Over 50% of the PA measured fluorometrically was found to be Phe and in groups (c), (d), and (e) the plasma Phe levels were in the range associated with a high risk of fetal damage in pregnant hyperphe women (Tables 6 and 13).

Body Weight: Figure 6 illustrates the mean daily body weight during the 25 days of the experiment for the animals in groups (a) through (e). Animals in the 3.5% Phe group (a) continued to gain weight at a steady rate throughout the experiment. Animals consuming diets containing both 3.5% Phe and pCPA lost weight during the first 3 to 5 days of the experiment. The weight loss in the 3.5% Phe 0.75% pCPA group can be accounted for by the loss of weight by one animal which refused to eat this diet after the first day of the experiment; this guinea pig had to be removed from the group on day 3 of the experiment. By the fifth day of the experiment, the mean daily body weights no longer declined in any group, however animals in the 3.5% Phe 1.25% pCPA and 3.5% Phe 1.50% pCPA groups did not regain their initial weight losses. Animals in the 3.5% Phe 0.75% pCPA

Figure 6. Series C, Experiment 1. Mean daily body weight (g) of each test diet group.

(a) 3.5% Phe
(b) 3.5% Phe 0.75% pCPA
(c) 3.5% Phe 1.0% pCPA
(d) 3.5% Phe 1.25% pCPA
(e) 3.5% Phe 1.50% pCPA

Figure 7. Series C, Experiment 1. Mean daily food intake (g/24 hour) of each test diet group. Groups (a) through (e) illustrated as in Figure 6.

Figure 6

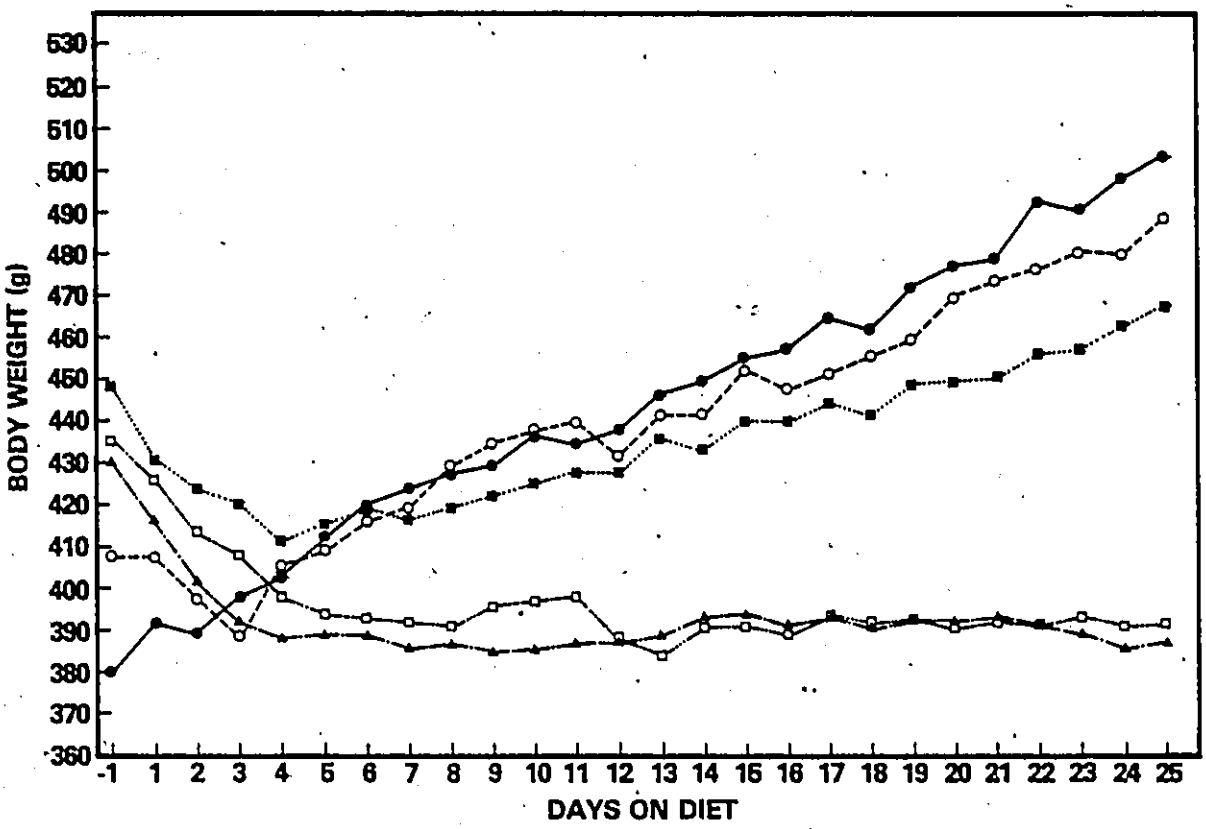
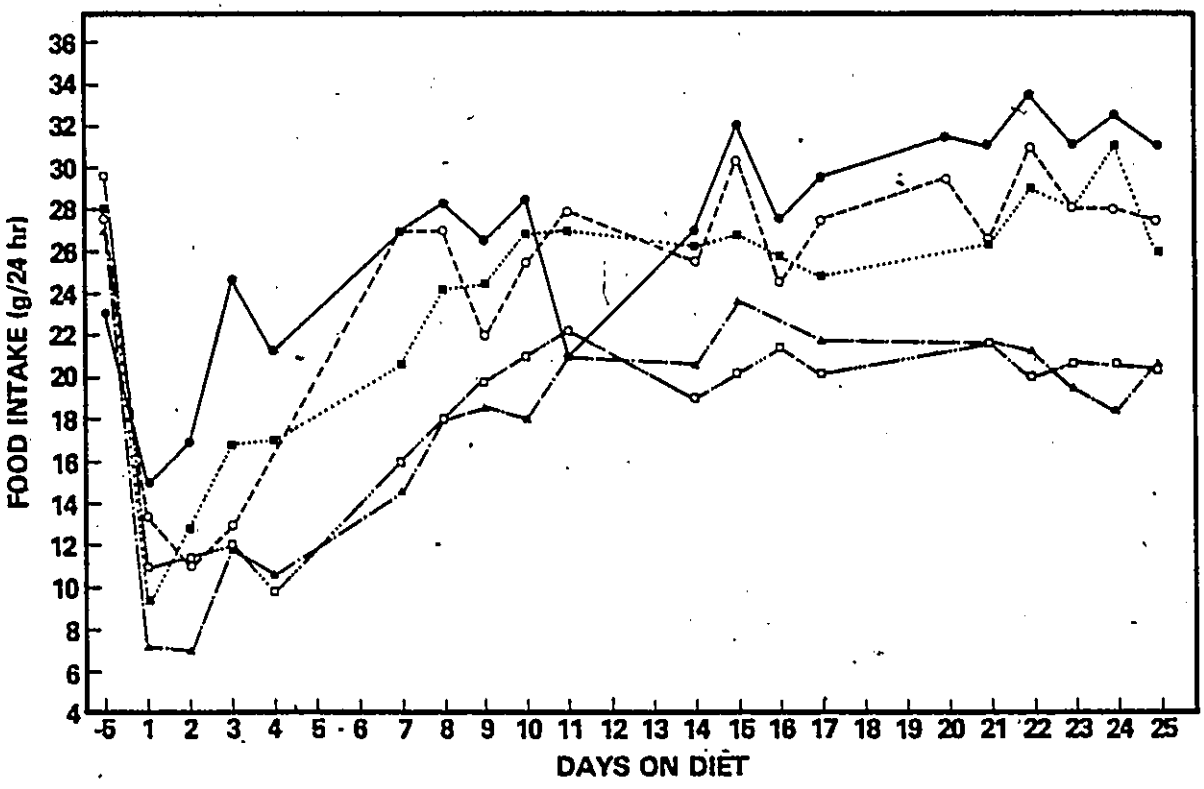


Figure 7



and 3.5% Phe 1.0% pCPA groups gained weight at a steady rate, although the latter group grew more slowly (Figure 6).

Food Intake: The initial weight loss and poor subsequent growth in the groups consuming Phe and pCPA diets may be related, at least in part, to the food intake of these animals (Figure 7). During the first 2 days of the test diet period the mean daily food intake of all groups fell to one half to one quarter of pretest diet levels. Food intake in the 3.5% Phe, 3.5% Phe 0.75% pCPA, and 3.5% Phe 1.0% pCPA groups gradually returned to the normal range; however after day 15 food intake of the animals in the groups consuming 1.25% pCPA and 1.50% pCPA supplemented diets stabilized at about two thirds of prediet levels (Figure 7). The food intake data of groups (a) through (e) during the last 10 days of the experiment were subjected to ANOVA with repeated measures. This ANOVA did not detect a significant effect for the test diet day ($F = 1.233$, $df = 6, 84$); however a highly significant effect for the test diet group was detected ($F = 6.631$, $df = 4, 14$, $p < .005$). Student-Neuman-Keuls tests indicated that the food intake in groups (d) and (e) was significantly lower ($p < .05$) than group (a). Table 14 shows the mean food intake for groups (a) through (e) over the final 10 days of the experiment as well as the intake of Purina chow for each of these groups prior to the test diet period. Paired t-tests (two-tailed) were used to compare the mean food intake during the final 10 days of the

Table 14 Series C, Experiment 1 Mean daily food intake (g/24 hours) during the final 10 days of the test diet period and pretest diet (Purina chow). Pooled prediet is the mean intake for all animals while eating Purina chow. Paired t-test were done comparing the mean intake of each animal during the final 10 days of the test diet period with the prediet intake of Purina. Unpaired t-tests compared the final 10 days test diet intake with the pooled prediet intake of all animals.

Diet Group	FOOD INTAKE (g/24 hour) Purina Chow Pre Test Diet Mean ±S.D.	Test Diet Period final 10 days Mean ±S.D.	t-tests (two-tailed) Paired vs prediet t	df	p*	Unpaired vs pooled prediet t	df	p*
All-Pooled Prediet Purina	27.6±3.7							
(a) 3.5% Phe	23.0±1.4	30.8±1.6	52.33	1	< 0.02	1.18	17	N.S.
(b) 3.5% Phe 0.75% pCPA	27.5±0.7	27.6±6.1	0.021	1	N.S.	0.016	17	N.S.
(c) 3.5% Phe 1.0% pCPA	28.0±2.9	27.2±3.8	1.11	4	N.S.	0.225	20	N.S.
(d) 3.5% Phe 1.25% pCPA	29.6±2.3	20.7±3.2	4.15	4	< 0.02	3.80	20	< 0.01
(e) 3.5% Phe 1.50% pCPA	27.0±7.0	20.8±2.3	1.21	2	N.S.	4.06	20	< 0.001

*p = probability, N.S. = not significant

test diet period with the pretest diet Purina chow intake of each group. Group (a) consumed significantly more food during the test diet period while group (d) ate significantly less (Table 14). When the test diet intake was compared to the pooled pretest diet Purina chow consumption of all groups, both groups (d) and (e) were found to have significantly decreased test diet consumption (Table 14).

The food intake data indicate that during the final 10 days of the test diet period the intake of all groups stabilized and that groups (d) and (e) ate significantly less compared to both group (a) and to pretest diets Purina chow consumption. In addition the food intake dropped sharply in all test diet groups when the guinea pigs were initially presented with the Phe and pCPA supplemented test diets (Figure 7).

Series C, Experiment 2

Introduction

Series C, Experiment 1 demonstrated that guinea pigs fed diets supplemented with 3.5% Phe and varying amounts of pCPA have elevated plasma levels of PA and Phe which were maintained for at least 23 days. Plasma Tyr was only moderately elevated in these animals, although the groups fed 3.5% Phe test diets which also contained pCPA had lower mean Tyr levels than animals fed a 3.5% Phe test diet without pCPA. These higher Tyr levels suggest that animals fed pCPA test diets converted Phe to Tyr less efficiently than did the animals not given pCPA, and inhibition of Phe hydroxylase activity in the livers of guinea pigs fed pCPA test diet was in fact demonstrated. A dietary supplement of 1.0% pCPA was associated with the lowest hepatic Phe hydroxylase activity. In addition, supplementation of the test diets with greater than 1.0% pCPA did not increase plasma Phe levels but was associated with significantly lower food intake and poor weight gain. Based on these data 1.0% pCPA was selected as the optimum dietary pCPA supplement.

In the following experiment the most effective dietary Phe supplement was determined. Guinea pigs were

fed test diets supplemented with 1.0% pCPA and varying amounts of Phe. Plasma levels of PA, Phe, and Tyr as well as body weight and food intake were monitored in these animals.

Methods

All animals used were nonpregnant adult female guinea pigs of the Hartley strain. All diets were prepared as described above and were supplemented with 1.0% pCPA and varying amounts of Phe. During the experiments animals were weighed daily and food intake was calculated in g per 24 hour. The animals were bled between 0900 and 1200 hours both before the test diets were given and at intervals during the test diet periods. Heparinized plasma was collected and assayed fluorometrically for PA, Phe, and Tyr. When sufficient plasma was available Phe and Tyr were also estimated by paper chromatography.

The animals were allocated to 9 test diet groups as summarized in Table 15.

Group (a), 2.5% Phe 1.0% pCPA diet: Seven guinea pigs, 55 days of age, were given a diet supplemented with 2.5% Phe 1.0% pCPA, 5% sucrose and 5% lettuce. Initially the animals were not given daily ascorbic acid supplements; data from this portion of the study are included in Series D, Experiment 1. After 15 and 16 days without ascorbic acid supplements, 4 animals and 3 animals, respectively, were

Table 15 Series C, Experiment 2. Experimental design summary.*

GROUP	DIET*	GUINEA PIGS			Number of Days on Test Diet	Route of Ascorbic Acid
		Number	Age (days)	Supplier		
(a)	2.5% Phe 1.0% pCPA 5% suc 5% cab	7	55	Carworth	22	IP
(b)	3.0% Phe 1.0% pCPA 5% suc 5% cab	3	79	Carworth	24	IP
(c)	3.5% Phe 1.0% pCPA 5% suc 5% cab	3	152	Carworth	21	IP
(d)	3.5% Phe 1.0% pCPA 7% suc 3% cab	3 2	112 75	Sunrise Mousery	23	oral
(e)	3.5% Phe 1.0% pCPA 8% suc	4	59	M & L Farms	29	oral
(f)	3.75% Phe 1.0% pCPA 8% suc	6	57	M & L Farms	31	oral
(g)	4.0% Phe 1.0% pCPA 8% suc	6	57	M & L Farms	31	oral
(h)	4.25% Phe 1.0% pCPA 8% suc	6	57	M & L Farms	31	oral

Continued...

Table 15 (Con't) Series C, Experiment 2

GROUP	DIET*	GUINEA PIGS			Number of Days on Test Diet	Route of Ascorbic Acid
		Number	Age (days)	Supplier		
(i)	1.0% pCPA 5% suc 5% cab	4	79 79 131 155	Carworth	24 24 3 26	IP
	4% suc 5% cab	1	184	Carworth	22	IP
	7% suc 3% cab	1	129	Sunrise Mousery	7	oral

* suc = sucrose
cab = cabbage

IP = intraperitoneal

given daily IP injections of freshly prepared ascorbic acid (40 mg/300 g body weight, neutralized, 2 ml/300 g body weight) and a diet supplemented with 2.5% Phe 1.0% pCPA 5% sucrose and 5% cabbage. The animals were bled by the intraorbital method 2, 7, 10, 14, 17, and 21 days after the ascorbic acid supplements began. Sufficient plasma for duplicate fluorometric analysis was available only for the samples collected on day 7.

Group (b), 3.0% Phe 1.0% pCPA diet: Following the conclusion of the study just described, three of these animals were given Purina chow ad libitum for 3 days and then given a diet supplemented with 3.0% Phe 1.0% pCPA 5% sucrose and 5% cabbage. The animals were 79 days of age when given the 3.0% Phe 1.0% pCPA diet. Ascorbic acid was administered daily (40 mg/300 g IP). Animals were bled by the intraorbital method 4, 7, 11, 14, 18, 21, and 24 days after receiving the 3.0% Phe 1.0% pCPA test diet.

Group (c), 3.5% Phe 1.0% pCPA diet: Three animals included in groups (a) and (b) were given a 3.5% Phe 1.0% pCPA 5% sucrose 5% cabbage diet when 152 days of age. The animals had been eating Purina chow ad libitum for 6 days prior to the initiation of this experiment. While consuming the 3.5% Phe 1.0% pCPA diet all animals received daily IP injections of ascorbic acid (40 mg/300 g). Animals were bled by the intraorbital method 3, 8, 11, 15, 18, and 21 days after the experiment began. On day 21 duplicate

fluorometric analyses were done on most samples.

Group (d), 3.5% Phe 1.0% pCPA diet: The data obtained from the five animals fed the 3.5% Phe 1.0% pCPA 7% sucrose 3% cabbage test diet in Series C, Experiment 1 are included here for purpose of comparison.

Group (e), 3.5% Phe 1.0% pCPA diet: This group was included to determine the effect of dietary cabbage supplement and the route of ascorbic acid supplement on plasma PA, Phe, and Tyr levels. Four guinea pigs, obtained from M. & L. Farms*, Flemington, New Jersey, were given a 3.5% Phe 1.0% pCPA 8% sucrose diet when 59 days of age. During the first 5 days of the experiment the animals were given oral ascorbic acid (50 mg/300 g body weight) freshly dissolved in sucrose solution (4 g/50 ml); after this time the supplement was divided into a morning and a late afternoon dose of 25 mg/300 g. As part of another study, (Series C, Experiment 3), blood was collected from a number of sources, including the eye (intraorbital) and the saphenous vein. The latter method, which avoids the use of anesthesia, involved shaving of the posterior aspect of the hind limb to permit visualization of the large subcutaneous saphenous vein. The skin was cleaned with 70% ethyl alcohol and a small incision exposing the surface of the vein was made. The vein was then carefully nicked, but not severed, and blood collected

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* M. & L. Farms had taken over the Carworth breeding stock, so the animals used in this and most subsequent experiments were from the same breeding population.

with a heparinized fine-tipped pipette. When approximately 0.7 ml had been obtained, bleeding was stopped by the application of slight pressure, and the wound again washed with 70% ethyl alcohol. Infection following this procedure was exceedingly uncommon. Plasma was collected from both the eye and the leg vein 6, 12, 15, 19, 22, 26 and 29 days after the test diet was introduced. Only the data from blood obtained from these two sources are included in the present experiment. Samples were analyzed in duplicate by fluorometry, and when sufficient plasma was available, also by paper chromatography.

Group (f), 3.75% Phe 1.0% pCPA diet: Six guinea pigs obtained from M. & L. Farms were given a 3.75% Phe 1.0% pCPA 8% sucrose test diet when 57 days of age. During the test diet period the animals received daily oral ascorbic acid supplements (50 mg/300 g body weight). Except on days 3, 4, and 5 of the experiment, the daily dose was divided into a morning and afternoon dose of 25 mg/300 g. Eight (8) days after the test diet was introduced the animals were bled from both the leg vein and the eye. Subsequently the animals were bled from the leg vein on days 13, 17, 21, 24, and 31 of the study. Samples were assayed fluorometrically in duplicate and by paper chromatography when sufficient plasma was available.

Group (g), 4.0% Phe 1.0% pCPA diet: When 57 days of age six animals obtained from M. & L. Farms were given a

test diet supplemented with 4.0% Phe 1.0% pCPA and 8% sucrose. The animals were handled identically to those in group (f) except they were given a diet supplemented with 4.0% Phe.

Group (h), 4.25% Phe 1.0% pCPA diet: The six animals in this group were identical to those in group (g) except that they were given a diet supplemented with 4.25% Phe 1.0% pCPA and 8% sucrose and the blood collected on day 8 of the experiment was obtained only from the eye. Groups (e), (f), (g), and (h) were run concurrently and had been received in the laboratory in the same shipment from M. & L. Farms.

Group (i), 1.0% pCPA diets: Six guinea pigs, ranging in age from 79 days to 184 days, were included in this group (Table 15). Five animals were purchased from Carworth and one from Sunrise Mousery. Four animals were given diets containing 1.0% pCPA, 5% sucrose and 5% cabbage for up to 26 days. One guinea pig was given a diet supplemented with 1.0% pCPA, 4% sucrose and 5% cabbage for a period of 22 days, and the remaining guinea pig ate a 1.0% pCPA 7% sucrose 3% cabbage diet and was included in Series C, Experiment 1. Except for the latter animal, the guinea pigs had consumed other experimental diets prior to the 1.0% pCPA diets and in this experiment were given daily IP ascorbic acid (40 mg/300 g/day). The animal included in Series C, Experiment 1 received oral ascorbic acid (50 mg/300 g/day). Plasma was collected by the intraorbital method at various times during

the test diet period.

In groups (e), (f), (g), and (h) $FeCl_3$ tests were done when fresh urine was available; fresh urine was collected when animals voided while being bled or weighed.

In addition to the fluorometric and paper chromatographic analyses, it was possible to measure plasma Phe, Tyr, and pCPA levels by column chromatography using a Beckman 120C Amino Acid Analyzer. Four plasma samples obtained from animals consuming diets supplemented with 3.5% Phe and 1.0% pCPA were run on the column. One sample was collected from the eye of an animal in group (d) after 25 days on the test diet and one was taken from the leg vein of an animal in group (e) after 15 days on the diet. Two additional samples were obtained from the leg veins of animals consuming 3.5% Phe 1.0% pCPA test diets for 24 days as part of a subsequent study (Series C, Experiment 4). One animal was bled at 0943 and 1340 hours, and the plasma pooled for this analysis, and the second animal was bled at 1331 and 1727 hour and the plasma pooled for analysis. Plasma was also collected for column chromatography from two guinea pigs in each of groups (f), (g), and (h). Three additional plasma samples obtained from three animals fed only Purina chow (1 male, 2 female) were also run on the column.

Results

Plasma PA, Phe, Tyr: The fluorometric determination

of plasma PA and Tyr for all groups in the experiment, except (d), are summarized in Tables 16 through 23; the PA and Tyr data for group (d) are included in Table 12. All groups were found to have a persistent elevation of plasma PA during the test diet period. Mean plasma Tyr levels were substantially elevated in group (h) but only moderately elevated in the other groups. Figure 8 illustrates the average PA and Tyr levels during the test diet period for each diet group.

Animals consuming the 2.5% Phe 1.0% pCPA test diet, (group a), had mean PA levels between 10.6 and 19 mg/100 ml during the first 18 days of the experiment. PA levels fell to 7.2 mg/100 ml on the final day of the study (Table 16). The mean PA level during the entire study was 14.5 mg/100 ml. Mean Tyr levels did not rise above 3.4 mg/100 ml and averaged 2.5 mg/100 ml over the course of the experiment (Table 16).

An increase in the dietary supplement of Phe to 3.0% (group b) did not substantially increase plasma PA levels above those observed in group (a) (Table 17, Figure 8). The mean PA level was 14 mg/100 ml for the duration of the study in group (b) and daily levels ranged from 7.9 to 16.6 mg/100 ml. Plasma Tyr levels averaged 3.3 mg/100 ml, slightly above the mean Tyr of animals consuming the 2.5% Phe 1.0% pCPA diet (Figure 8).

Animals in groups (c), (d), and (e) were fed test

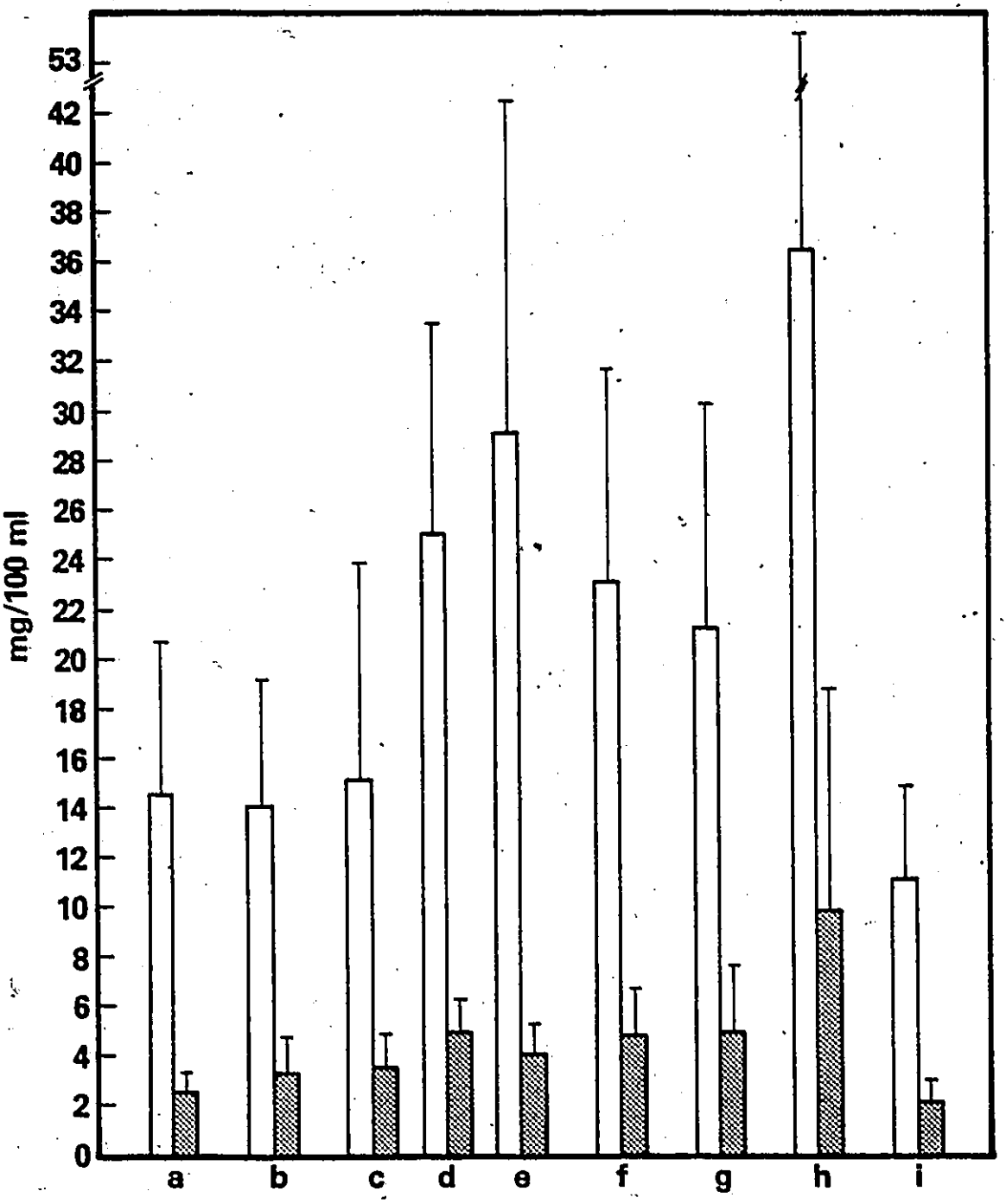


Figure 8. Series C, Experiment 2. Mean plasma total phenylalanines (open bars) and tyrosine (shaded bars) of each diet group over the duration of the experiment. Fluorometric method. Error bars indicate standard deviation.

Table 16 Series C, Experiment 2, Group (a). Plasma phenylalanines and tyrosine as a function of number of days on diet, 2.5% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

	NUMBER OF DAYS ON DIET (2.5% Phe 1.0% pCPA)							
	Prediet (Purina)	3	8	11	15	18	22	TOTAL (on test diet)
Number of Samples	6	4	7	7	7	7	7	39
Mean Phenylalanines	0.90	16.50	19.03	10.64	19.03	15.24	7.19	14.46
±S.D.	±0.13	±3.63	±6.89	±1.66	±5.69	±4.20	±2.23	±6.19
Mean Tyrosine	0.67	2.30	3.39	2.16	2.79	2.54	1.76	2.50
±S.D.	±0.41	±0.35	±1.34	±0.30	±0.73	±0.53	±0.43	±0.87
Phenylalanines/ Tyrosine	2.43	7.17	5.61	4.93	6.82	6.00	4.09	5.78

Table 17 Series C, Experiment 2, Group (b). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 3.0% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

		NUMBER OF DAYS ON DIET (3.0% Phe 1.0% pCPA)								
		Prediet (Purina)	4	7	11	14	18	21	24	TOTAL (on test diet)
Number of Samples		2	3	3	3	3	3	3	3	21
Phenylalanines	Mean	0.80	15.67	15.63	13.80	7.93	12.70	15.97	16.59	14.04
	±S.D.	±0.14	±10.51	± 3.81	± 4.33	± 1.44	± 1.14	± 3.55	± 5.33	± 5.19
Tyrosine	Mean	1.00	3.10	5.30	2.67	2.03	2.87	3.07	4.03	3.29
	±S.D.	±0.71	± 1.90	± 2.07	± 0.42	± 0.29	± 0.23	± 0.21	± 2.12	± 1.52
Phenylalanines/ Tyrosine		1.10	5.05	2.95	5.17	3.91	4.43	5.20	4.11	4.27

diets supplemented with 3.5% Phe and 1.0% pCPA. During the first 18 days of the study, animals in group (c) had mean PA levels ranging from 12.8 mg/100 ml to 21.1 mg/100 ml. On day 21 however, the mean PA level fell to 6.5 mg/100 ml (Table 18). This low PA level may reflect, at least in part, the fact that due to a miscalculation, on the morning blood was collected these animals had essentially run out of test diet. PA averaged 15.2 mg/100 ml over the 21 test diet days, (PA averaged 16.9 mg/100 ml when the day 21 samples were excluded). Mean daily Tyr levels ranged from 2.4 mg/100 ml to 4.2 mg/100 ml, and averaged 3.5 mg/100 ml during the entire study (Table 18, Figure 8).

The data from group (d) were discussed above (Series C, Experiment 1, Table 12). Over the course of the entire experiment PA and Tyr levels averaged 25 mg/100 ml and 4.9 mg/100 ml, respectively (Figure 8).

The plasma PA and Tyr data from group (e) are summarized in Table 19. Mean PA levels ranged from 16.5 to almost 43 mg/100 ml while mean Tyr levels were between 2.3 mg/100 ml and 6.3 mg/100 ml. The mean PA and Tyr levels for the duration of the study were about 29 mg/100 ml and 4 mg/100 ml, respectively (Table 19). On most occasions blood was obtained from both the ophthalmic venous plexus and the saphenous vein (Table 19). Plasma was available for fluorometric analysis from 19 pairs of blood samples collected from both sources. Paired t-tests were used to

Table 18 Series C, Experiment 2, Group (c). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 3.5% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

		NUMBER OF DAYS ON DIET (3.5% Phe 1.0% pCPA)								
		Prediet (Purina)	3	4	8	11	15	18	21	TOTAL (on test diet)
Number of Samples		2	2	1	3	3	3	3	3	18
Phenylalanines	Mean	1.00	18.95	18.3	18.13	13.67	21.07	12.80	6.50	15.15
	±S.D.	0	± 7.14		± 2.45	± 5.12	±13.21	±13.93	± 2.12	± 8.71
Tyrosine	Mean	0.50	3.95	2.8	3.60	3.27	4.17	3.77	2.40	3.46
	±S.D.	0	± 0.71		± 1.25	± 1.70	± 2.02	± 1.86	± 1.22	± 1.40
Phenylalanines/ Tyrosine		2.00	4.80	6.54	5.04	4.18	5.05	3.40	2.71	4.38

Table 19 Series C, Experiment 2, Group (e). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 3.5% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

Source of Blood		NUMBER OF DAYS ON DIET (3.5% Phe 1.0% pCPA)								
		Predlet (Purina)	6	12	15	19	22	26	29	Total (on test diet)
Leg Vein	Number of Samples	2	4	4*	4	4	4		1	21*
	Phenylalanines Mean	1.55	24.95	36.05	27.22	29.72	31.85		12.1	29.11
	Phenylalanines ±S.D.	±0.07	±12.94	±25.46	±7.31	±9.19	±6.38			±13.38
	Tyrosine Mean	0.60	3.35	4.80	5.35	4.18	2.82		1.9	3.96
	Tyrosine ±S.D.	±0.85	±0.99	±1.44	±0.79	±0.72	±0.75			±1.32
	Phenylalanines / Tyrosine	2.58	7.45	7.51	5.09	7.11	11.29		6.37	7.35
Eye	Number of Samples	4	3	4**	4	4	4	4	4	27**
	Phenylalanines Mean	1.75	29.60	42.95	33.32	30.92	32.30	17.80	16.48	29.03
	Phenylalanines ±S.D.	±0.17	±7.70	±29.37	±9.32	±7.55	±6.59	±7.69	±7.83	±14.77
	Tyrosine Mean	1.75	2.37	4.60	6.32	4.12	2.78	4.15	2.48	3.82
	Tyrosine ±S.D.	±0.66	±0.55	±1.98	±1.36	±1.11	±0.51	±1.79	±1.26	±1.76
	Phenylalanines / Tyrosine	1.00	12.49	9.34	5.27	7.50	11.02	4.29	6.65	7.60

* one sample not analyzed for tyrosine.
** two samples not analyzed for tyrosine.

determine if plasma PA and Tyr levels differed significantly in blood from these two sources on days 6, 12, 15, 19, and 22; none of these tests reached statistical significance at the 0.05 level. The mean PA levels of plasma from the eye and the leg vein for these paired samples were 33.53 ±14.97 mg/100 ml and 30.65±13.14 mg/100 ml, respectively. The significance of this difference in PA levels was assessed using a paired t-test and found to be of only marginal significance ($t = 2.136$, $df = 18$, $0.025 > p < 0.05$, two-tailed). The mean Tyr levels during the study, 3.84 ±1.82 mg/100 ml for the eye bloods and 3.95±1.25 mg/100 ml for the leg vein bloods, did not differ significantly (paired $t = 0.554$, $df = 18$, two-tailed).

Table 20 summarizes the plasma PA and Tyr data of the animals fed the 3.75% Phe 1.0% pCPA test diet, group (f). Mean PA levels ranged from 17.1 mg/100 ml to 35.45 mg/100 ml and averaged about 23 mg/100 ml during the whole experiment. Mean Tyr levels ranged from 3.5 mg/100 ml to 5.7 mg/100 ml and averaged 4.7 mg/100 ml; one sample obtained on day 21 had a Tyr concentration of 11.0 mg/100 ml. On day 8 of the experiment blood was collected from both the eye and the leg vein (Table 20). Paired t-tests did not reveal significant differences in PA and Tyr levels in plasma from the two sources (PA: $t = 1.729$, $df = 5$ two-tailed; Tyr: $t = 0.468$, $df = 5$, two-tailed).

Table 20 Series C, Experiment 2, Group (f). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 3.75% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

		NUMBER OF DAYS ON DIET (3.75% Phe 1.0% pCPA)									
		Prediet (Purina)		8		13	17	21	24	31	TOTAL (on test diet)
Source of Blood		Eye	Leg	Eye	Leg	Leg	Leg	Leg	Leg	Leg	Leg
Number of Samples		6	2	6	6	5	5	5	5	2	28
Phenylalanines	Mean	1.67	1.40	22.28	21.12	17.14	22.88	25.84	24.22	35.45	23.14
	±S.D.	±0.29	0	±11.43	±10.45	±5.91	±7.98	±10.23	±3.69	±5.02	±8.58
Tyrosine	Mean	1.30	1.20	4.72	5.03	3.48	5.18	5.74	3.70	5.00	4.67
	±S.D.	±0.33	±0.28	±2.34	±2.36	±1.11	±1.53	±3.08	±1.12	±1.70	±2.00
Phenylalanines/ Tyrosine		1.28	1.17	4.72	4.20	4.93	4.42	4.50	6.55	7.09	4.96

The animals in group (g) consumed a test diet supplemented with 4.0% Phe and 1.0% pCPA. Mean PA levels ranged from 14.3 mg/100 ml to 26.9 mg/100 ml and averaged 21.2 mg/100 ml over the whole study period (Table 21). Mean Tyr levels ranged from 2.8 mg/100 ml to 5.5 mg/100 ml and averaged 4.9 mg/100 ml for the whole experiment. Although the overall mean Tyr level of the animals in group (g) is comparable to those of groups (c), (d), (e) and (f), one animal had 5 plasma samples during the test diet period which ranged between 8.0 and 10.6 mg/100 ml Tyr. On day 8 of the study blood was collected from both the ophthalmic plexus and the saphenous vein (Table 21). Comparison of PA and Tyr levels in plasma from the two sources by paired t-tests did not reveal statistically significant differences (PA: $t = 0.764$, $df = 4$, two-tailed; Tyr: $t = 3.011$, $df = 4$, two-tailed).

Table 22 summarizes the mean plasma levels of PA and Tyr of the animals consuming the 4.25% Phe 1.0% pCPA diet, group (h). Mean PA levels ranged from 25.4 mg/100 ml to over 50 mg/100 ml and averaged 36.4 mg/100 ml during the whole experiment. Except for test diet days 8 and 24, mean Tyr levels were substantially elevated relative to both prediet levels and those of animals consuming diets containing less than 4.25% Phe. Mean Tyr levels were never less than 5 mg/100 ml and exceeded 13 mg/100 ml on two days (Table 22). Plasma Tyr averaged 9.8 mg/100 ml during the

Table 21 Series C, Experiment 2, Group (g). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 4.0% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

		NUMBER OF DAYS ON DIET (4.0% Phe 1.0% pCPA)							
		Prediet (Purina)	8	13	17	21	24	31	TOTAL (on test diet)
Number of Samples		6*	6	6*	6	6**	6	5	1	30**
Phenylalanines	Mean	1.45	18.58	19.08	14.28	20.92	26.90	24.06	32.3	21.22
	±S.D.	± 0.19	± 6.50	± 6.17	± 5.44	± 8.19	±10.05	±11.42		± 9.11
Tyrosine	Mean	1.46	4.48	2.78	4.43	4.50	5.52	4.50	10.6	4.89
	±S.D.	± 0.78	± 2.96	± 1.74	± 3.10	± 1.86	± 2.68	± 2.72		± 2.71
Phenylalanines/ Tyrosine		0.99	4.15	6.86	3.22	4.65	4.87	5.35	3.05	4.34

* Blood obtained from the eye, not included in TOTAL

** One sample obtained from the eye.

Table 22 Series C, Experiment 2, Group (h). Plasma phenylalanines and tyrosine as a function of the number of days on diet, 4.25% Phe 1.0% pCPA, fluorometric analyses, mg/100 ml.

	NUMBER OF DAYS ON DIET (4.25% Phe 1.0% pCPA)							
	Prediet (Purina)	8	13	17	21	24	31	TOTAL (on test diet)
Number of Samples	5*	4*	6	6	6	6	2	30
Mean Phenylalanines	1.32	59.22	36.98	30.58	37.02	25.35	38.50	36.45
±S.D.	±0.23	±13.32	±25.06	±9.74	±10.52	±6.60	±16.25	±16.83
Mean Tyrosine	1.46	5.10	9.30	13.73	12.57	5.20	13.85	9.76
±S.D.	±0.68	±2.23	±8.37	±13.58	±10.29	±4.49	±2.76	±8.93
Phenylalanines/ Tyrosine	0.90	11.61	3.98	2.23	2.95	4.88	2.78	3.37

* Blood obtained from the eye.

whole experiment. Eleven plasma samples, from 5 different animals, were found to have Tyr concentrations greater than or equal to 8 mg/100 ml; 8 of these exceeded 11 mg/100 ml and 2 were over 30 mg/100 ml. The marked elevation of plasma Tyr among the animals eating 4.25% Phe 1.0% pCPA is reflected by the relatively low PA:Tyr ratios when compared to the other test diet groups (Tables 12 and 16 to 23).

Animals consuming diets supplemented with 1.0% pCPA had a mean plasma PA level of 11.1 mg/100 ml and a mean Tyr level of 2.0 mg/100 ml (Table 23). The variation in plasma PA, as indicated by the standard deviation (S.D.), is considerably less than that observed in each of the other groups in this experiment (Tables 12, and 16 to 23). Plasma Tyr levels were the lowest observed among the test diet groups and on most occassions were within the normal range (Table 23).

The fluorometric plasma PA data from the test diet period were transformed to logarithms and subjected to ANOVA with repeated measures. For this analysis the PA data from animals consuming 3.5% Phe 1.0% pCPA diets, groups (c), (d) and (e), were pooled. In order to assess the effect of the duration of test diet consumption on plasma PA levels, the data from all groups were assembled into 4 time intervals, days 3-8, days 11-15, days 17-19, and days 21-26. This permitted comparison of the PA levels of

Table 23 Series C, Experiment 2, Group (i). Plasma phenylalanines and tyrosine as a function of number of days on diet, 1.0% pCPA, fluorometric analyses, mg/100 ml.

	NUMBER OF DAYS ON DIET (1.0% pCPA)										
	Prediet (Purina)	3	4	5	6	7	8	11	12	14	15
Number of Samples	6	2	1	1	1	2	2	2	2	2	2
Phenylalanines Mean	1.45	10.8	10.4	20.5	16.2	12.3	8.1	9.3	13.8	6.3	8.2
Phenylalanines S.D.	0.90	2.0				4.0	3.8	1.6	0.6	0.1	6.2
Tyrosine Mean	0.83	2.1	1.8	2.1	1.8	2.6	1.7	1.4	4.0	1.6	2.2
Tyrosine S.D.	0.52	0				0.6	1.4	0.1	2.3	0.2	0.5
Phenylalanines/ Tyrosine	1.75	5.14	5.78	9.76	9.0	4.73	4.76	6.64	3.45	3.94	3.73

Continued...

Table 23 (Con't)

	NUMBER OF DAYS ON DIET (1.0% pCPA)						
	18	19	21	22	24	26	Total (on test diet)
Number of Samples	3	1	2	2	2	1	28
Phenylalanines Mean	9.47	12.3	12.2	14.4	10.4	11.5	11.10
Phenylalanines S.D.	1.96		2.8	4.7	0.8		±3.70
Tyrosine Mean	1.47	2.4	2.2	2.3	1.9	0.6	2.04
Tyrosine S.D.	0.30		1.2	0.7	0.4		±0.91
Phenylalanines/ Tyrosine	6.44	5.13	5.55	6.26	5.47	19.17	5.44

the various groups after similar periods of test diet intake. When more than one blood sample was available from an animal during one of these time intervals, the values were averaged and the average taken as the PA level for that animal during the relevant time interval. Table 24 summarizes the plasma PA data as regrouped for this ANOVA. ANOVA revealed a significant interaction between the test diet day and the test diet group ($F = 2.652$, $df = 18, 104$ $p < .001$). This interaction suggests that during the test diet period the various groups had markedly different patterns of change (ie. slope) in plasma PA levels. Figure 8.1 illustrates these PA data and it is clear that the slopes of the PA concentration vs test diet day curves differ among groups. In order to determine whether there were significant differences in PA levels among the test diet groups the data for each test diet interval were subjected to one-way ANOVA. On days 3 to 8 ANOVA revealed a significant group effect ($F = 12.983$, $df = 6, 36$ $p < .0001$) and Student-Newman-Keuls multiple comparisons indicated that PA was significantly higher ($p < .05$) in group (h) compared to all other groups. The PA level of the 3.5% Phe 1.0% pCPA groups was significantly greater than the 1.0% pCPA group (i) ($p < .05$). No other significant differences were found on days 3 to 8. On day 11 to 15 the ANOVA also revealed a significant group effect ($F = 3.234$, $df = 6, 36$ $p = .012$) and the multiple comparison showed

Table 24 Series C, Experiment 2 Plasma total phenylalanines (PA) and tyrosine (Tyr) as measured fluorometrically. These data used for ANOVA with repeated measures.

DIET GROUP		DAYS 3-8 Mean ±S.D.**	DAYS 11-15 Mean ±S.D.	DAYS 17-19 Mean ±S.D.	DAYS 21-26 Mean ±S.D.	TOTAL Mean ±S.D.
(a)	N*	7	7	7	7	7
2.5% Phe	PA	18.01±5.30	14.86±3.34	15.24±4.20	7.19±2.23	13.82±5.52
1.0% pCPA	Tyr	3.04±1.04	2.47±0.43	2.54±0.54	1.76±0.43	2.45±0.78
(b)	N	3	3	3	3	3
3.0% Phe	PA	15.63±3.52	10.83±2.71	12.70±1.14	16.27±1.10	13.86±3.06
1.0% pCPA	Tyr	4.20±0.40	2.37±0.32	2.87±0.23	3.53±0.97	3.24±0.87
(c,d,e)	N	12	12	12	12	12
3.5% Phe	PA	25.03±6.45	23.98±14.98	24.22±12.46	22.84±9.86	24.02±11.04
1.0% pCPA	Tyr	3.62±1.41	4.25±1.48	4.21±1.48	4.06±1.93	4.03±1.56
(f)	N	5	5	5	5	5
3.75% Phe	PA	19.76±11.08	17.14±5.91	22.88±7.98	25.02±6.60	21.20±8.08
1.0% pCPA	Tyr	4.46±2.13	3.48±1.11	5.18±1.53	4.72±1.81	4.46±1.67
(g)	N	6	6	6	6	6
4.0% Phe	PA	18.58±6.47	14.28±5.44	20.92±8.19	26.47±10.61	20.06±8.65
1.0% pCPA	Tyr	4.48±2.96	4.43±3.10	4.50±1.86	5.07±2.40	4.62±2.46
(h)	N	6	6	6	6	6
4.25% Phe	PA	62.48±13.60	36.98±25.06	30.58±9.74	31.18±6.65	40.31±19.61
1.0% pCPA	Tyr	5.75±2.84	9.30±8.37	13.73±13.58	8.90±6.42	9.42±8.63
(i)	N	4	4	4	4	4
1.0% pCPA	PA	12.38±3.48	9.40±2.51	10.18±2.14	12.08±1.78	11.01±2.63
	Tyr	2.22±0.59	2.30±1.24	1.70±0.53	1.92±0.46	2.04±0.73

* number of animals; when more than one blood sample was collected from a single animal during this interval, the PA values were averaged and this average included in these data.

** mg/100 ml

Figure 8.1 Series C, Experiment 2. Mean plasma total phenylalanines on test diet days 3-8, 11-15, 17-19, and 21-26, illustrating the data used for ANOVA with repeated measures.

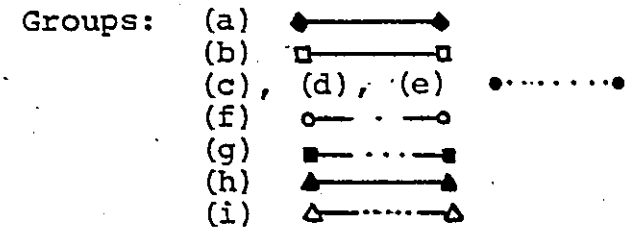
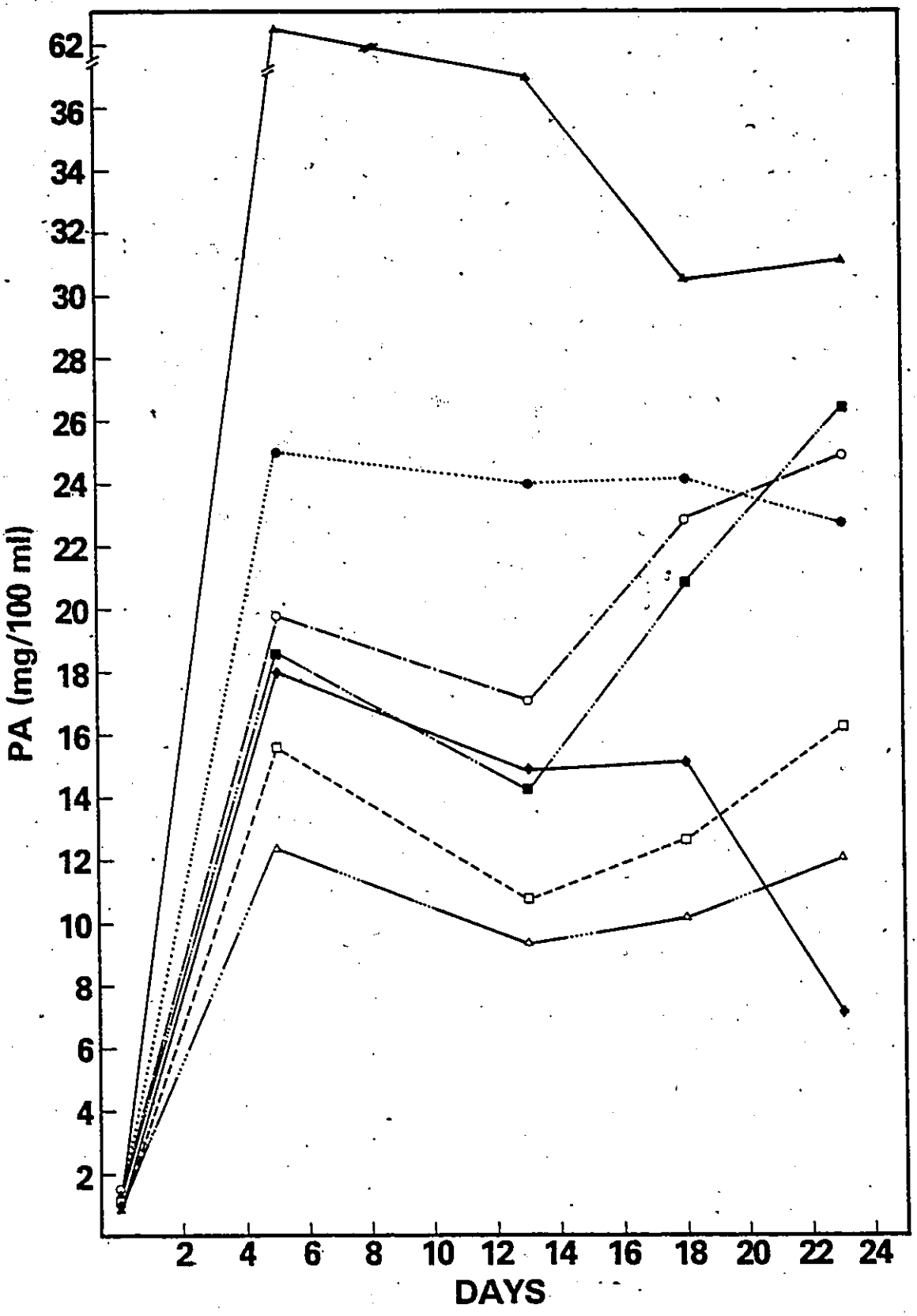


Figure 8.1


62
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
PA (mg/100 ml)
2
4
6
8
10
12
14
16
18
20
22
24
DAYS

only that group (h) had significantly higher PA levels than group (i) ($p < .05$). The ANOVA of the day 17 to 19 PA data did not detect a significant group effect ($F = 1.938$, $df = 6, 36$). The ANOVA was again significant on days 21-26. ($F = 8.510$, $df = 6,36$ $p < .001$). Multiple comparisons indicated that PA in group (h) was significantly greater than PA in groups (a) and (i) ($p < .05$). In addition the low PA in group (a) on day 22 (Table 16, Figure 18.1) was significantly lower than the PA in all other groups except (i) ($p < .05$). In summary these analyses show that the PA levels of group (h), 4.25% Phe 1.0% pCPA test diet, were significantly greater than those of the animals fed the 1.0% pCPA during three of the time intervals. In addition, the only other group to have significantly higher PA levels than the 1.0% pCPA group was the pooled 3.5% Phe 1.0% pCPA group.

Phe and Tyr were also estimated by paper chromatography. Table 25 includes the paper chromatographic and fluorometric data for the plasma samples analyzed by both methods.

Only 1 sample was chromatographed in each of groups (a) and (b). The Phe concentration was about 62% of the PA level in the plasma from the 2.5% Phe 1.0% pCPA animal and only 21% in the 3.0% Phe 1.0% pCPA sample (Table 25). It was not possible to do paper chromatography on other plasma samples from groups (a) and (b).

Phe constituted about 62% and 51% of the PA levels

Table 25 Series C, Experiment 2. Comparison (Mean ±S.D.) of fluorometric and paper chromatographic estimations of plasma phenylalanines and tyrosine[a]

DIET (Group)	N*	PHENLYLALANINES		TYROSINE	
		Fluorometric mg/100 ml	Paper Chromatographic mg/100 ml (% of fluorometric)	Fluorometric mg/100 ml	Paper Chromatographic mg/100 ml (% of fluorometric)
2.5% Phe 1.0% pCPA (a)	1	25.9	16 (61.8%)	3.1	<4 (64.5%)
3.0% Phe 1.0% pCPA (b)	1	14.0	<6 (21.4%)[c]	3.0	<4 (66.7%)
3.5% Phe 1.0% pCPA (c)	none				
3.5% Phe 1.0% pCPA (d)	11	25.35±7.97	15.8 ±8.2 (62.4%)	4.68±1.53	5.5 ±2.5 (117.5%)
3.5% Phe 1.0% pCPA (e)	19	24.16±9.69	12.3 ±10.2 (50.9%)	3.75±1.42	5.7 ±3.5 (151.5%)
3.75% Phe 1.0% pCPA (f)	9	26.57±8.53	14.1 ±6.9 (53.1%)	5.19±1.50	3.7 ±1.8 (71.7%)
4.0% Phe 1.0% pCPA (g)	9	21.07±7.07	9.1 ±5.8 (43.2%)	4.86±2.75	6.4 ±3.6 (131.5%)

Continued...

Table 25 Series C, Experiment 2 (Con't)

DIET (Group)	N	PHENLYLALANINES			TYROSINE		
		Fluorometric mg/100 ml	Paper Chromatographic mg/100 ml (% of fluorometric)		Fluorometric mg/100 ml	Paper Chromatographic mg/100 ml (% of fluorometric)	
4.25% Phe 1.0% pCPA (h)	10	32.54±14.55	17.8 ±9.3	(54.7%)	8.07±8.50	9.8 ±9.1	(120.8%)
1.0% pCPA (i)	2	13.60±3.68	3.0±0	(22.1%)	1.80±0	1.5 ±0.7	(83.3%)

* Number of samples analyzed by both methods.

** For purposes of calculation, chromatographic values of < 6, < 4, < 2 were taken as 3, 2, and 1 mg/100 ml, respectively.

in the two 3.5% Phe 1.0% pCPA diet groups, (d) and (e) (Table 25). Among the groups fed test diets supplemented with more than 3.5% Phe, the percentage of Phe in the PA did not rise above that of group (d), and only slightly exceeded the percentage Phe in group (e). Plasma Phe was in the range associated with significant risk to the human fetus in groups (d), (f), and (h). The paper chromatographic Phe data from groups (d), (e), (f), (g) and (h) were subjected to one-way ANOVA. The mean Phe level of each animal during the test diet period was used for this analysis and the data from groups (d) and (e) were pooled. This ANOVA revealed a barely significant test diet effect ($F = 3.097$, $df = 3, 21$ $p = .0488$). Student-Newman-Keuls tests could not detect significant differences between the groups, indicating that all the groups with at least 3.5% Phe added to the test diet had comparable elevations of plasma Phe.

Although the PA levels of the animals fed 1.0% pCPA test diets were clearly elevated, Phe was not detected in either plasma sample available for paper chromatography (Table 25). The Phe level of one of these samples was shown to be less than 3.0 mg/100 ml when twice the amount of plasma was run on paper and Phe still could not be detected.

In order to more accurately determine the concentration of Phe and pCPA, plasma samples were also analyzed by column chromatography. In addition to these data, Table 26 also includes the results of the fluorometric and paper

chromatographic analyses of these samples. Both the Phe and the pCPA concentrations as measured by paper and column chromatography were highest in the samples from animals in the 3.5% Phe 1.0% pCPA test diet groups. Phe averaged over 21 mg/100 ml in these samples and Phe therefore comprised about 60% of the PA levels measured fluorometrically (Table 26). The Phe levels determined by column chromatography were the lowest among the test diet groups in the samples from the 4.0% Phe 1.0% pCPA animals. In the 3.5% Phe 1.0% pCPA group, Phe levels measured by paper chromatography were higher than the levels determined by column chromatography in 3 of 4 samples, although there was better agreement between these methods in samples from the other test diet droups. Plasma pCPA levels tended to decline as the dietary Phe supplement increased, although the data are limited. Fluorometric and column chromatographic Tyr measurements compared well, and with the exception of the sample from the animal fed the 4.25% Phe 1.0% pCPA diet (Tyr 22 mg/100 ml) were similar among the test diet groups. The Tyr levels determined by paper and column chromatography were also simlar although one sample [Table 26: 3.5% Phe 1.0% pCPA group (d)], was considerably higher when estimated by paper chromatography.

The Tyr levels determined by fluorometry were also subjected to ANOVA with repeated measures. These Tyr data, like the PA data, were regrouped into four time

Table 26 Comparison of fluorometric, paper chromatographic, and column chromatographic determinations of plasma amino acids (mg/100 ml), effect of diet.

DIET	Group	PHENYLALANINES			p-Chlorophenyl-alanine	TYROSINE		
		Fluorometric	Column Chromato-graphy	Paper Chromato-graphy	Column Chromato-graphy	Fluorometric	Column Chromato-graphy	Paper Chromato-graphy
Purina	♂	2.2	1.14	<6	0		1.14	<4
	♀	1.8	0.97		0		1.01	
	♀	1.5	1.58	<6	0	1.2	1.07	<4
	Mean	1.83	1.23	<6	0	1.2	1.07	<4
3.5% Phe 1.0% pCPA	(d)	28.4	10.60	20	8.25	7.4	5.65	12
	(e)	37.5	16.86	20	7.70	6.0	4.71	4.5
	Exp 4	42.0 44.3	16.02	32 32	5.95	4.5 4.5	2.32	5 5
	Exp 4	32.8 37.8	42.00	24 24	7.79	4.0 2.6	3.92	5 <4
	Mean	36.23	21.37	24.0	7.42	5.30	4.15	6.25
3.75% Phe 1.0% pCPA	(f)	23.5	11.72		3.25	6.5	5.62	
	(f)	34.5	15.82		6.40	6.6	5.06	
	Mean	29.00	13.77		4.83	6.55	5.34	
4.0% Phe 1.0% pCPA	(g)	17.6	5.68	6	4.55	8.0	9.15	9
	(g)	12.8	4.71	<5	2.12	3.0	2.75	4.5
	Mean	15.20	5.20	4.5	3.34	5.50	5.95	6.75
4.25% Phe 1.0% pCPA	(h)	31.6	14.32		3.34	22.8	22.16	

intervals (Table 24) and transformed to logarithms for this analysis. A highly significant effect for the test diet group was detected ($F = 9.463$ $df = 6, 36$ $p << .001$). The Student-Newman-Keuls procedure showed that the 4.25% Phe 1.0% pCPA group had Tyr levels which were significantly greater than all other groups ($p < .05$). In addition, Tyr levels in groups (i) and (a) were significantly lower than all groups except group (b) ($p < .05$). Mean plasma Tyr levels over the whole test diet period increased as the dietary Phe supplement was increased (Table 24). The Tyr levels measured by paper chromatography in groups (d), (e), (f), (g) and (h) were subjected to one-way ANOVA (Table 25). The mean Tyr level for each animal during the test diet period was used and the data from the 3.5% Phe 1.0% pCPA groups were pooled. The ANOVA indicated a significant effect for the test diet ($F = 4.669$ $df = 3, 21$ $p = .012$). Student-Newman-Keuls tests showed that group (h) was again the highest and that Tyr levels in this group were significantly greater than those in group (f) ($p < .05$). In summary these analyses show that the test diet composition was a significant determinant of plasma Tyr levels and that the 4.25% 1.0% pCPA group had significantly higher Tyr levels (Tables 20, 24). In addition, the 1.0% pCPA and the 2.5% Phe 1.0% pCPA groups had the lowest Tyr levels while the remaining groups had moderately elevated Tyr levels which did not differ significantly from one another.

The 3.5% Phe 1.0% pCPA test diet was found to produce significant elevations of plasma PA and Phe. Approximately 60% of the PA levels in the 3.5% Phe 1.0% pCPA groups was composed of Phe, higher than any other test diet group. The hyperphe persisted for at least 29 days and was usually near 15 mg/100 ml. Only the 4.25% Phe 1.0% pCPA group had higher PA levels, this group however also had significantly higher Tyr levels. Tyr levels in the 3.5% Phe 1.0% pCPA animals were only moderately elevated. On the basis of the plasma PA, Phe, and Tyr data, the 3.5% Phe 1.0% pCPA diet was found to be most satisfactory.

$FeCl_3$ tests: A number of fresh urine samples from animals in groups (e) through (h) were tested with $FeCl_3$. The following number of specimens were tested from each group: (e) 1, (f) 6, (g) 6, and (h) 1. All samples were positive and tended to fade within a few minutes, indicating significant phenylketone excretion in these guinea pigs.

Body weight and food intake: The mean daily body weight and food intake of each group is summarized in Tables 27 and 28 respectively. The animals in group (a) gained weight and consumed food at a near-normal rate throughout the experiment. It is important to note that when the experiment began, these animals had been eating the 2.5% Phe 1.0% pCPA diet for 15 to 17 days (without ascorbic acid supplements) as part of another study (Series D, Experiment 1). These animals did in fact lose weight and

decrease food intake when initially given the 2.5% Phe 1.0% pCPA test diet in Series D (see below).

Animals consuming the 3.0% Phe 1.0% pCPA diet, group (b), lost about 2% of their mean body weight during the first 2 days on this diet (Table 27). After 9 days these animals gained weight at an average rate of 3.3 g/day. The early weight loss was paralleled by low food intake which subsequently increased to over 30 g/24 hour by day 10 (Table 28).

The guinea pigs in group (c) lost about 6% of their prediet body weight during the first 5 days of the 3.5% Phe 1.0% pCPA test diet period (Table 27). These animals maintained this weight until day 11 and then lost weight at a rate of about 2 g/day (Table 27). Although these animals were eating about 27 g/24 hours at the end of the study, they were also the oldest and heaviest in the experiment (Tables 15, 27), and were therefore consuming less diet than all other groups in g/kg body weight per day (42 g/kg/day vs a range of 61-72 g/kg/day at the conclusion of the study).

The animals in group (d) lost about 8% of their prediet body weight during the first 4 days on the 3.5% Phe 1.0% pCPA diet. They began to gain weight on the fifth day of the study at a rate of 2.7 g/day (Figure 6, Table 27). Food intake in group (d) dropped dramatically during the first day on the 3.5% Phe 1.0% pCPA diet and then began to increase on the second day (Table 28, Figure 7). From

Table 27 Series C, Experiment 2 Mean body weight (g) as a function of the number of days on diet.

DIET (Group)		NUMBER OF DAYS ON TEST DIET (continued)													
		Prediet	1	2	3	4	5	6	7	8	9	10	11	12	13
2.5% Phe	N*	7	7	7	7	7	7	7	7	7	7	7	7	7	7
1.0% pCPA	Mean	334.4	337.7	343.1	345.1	343.9	350.9	354.9	360.7	365.1	364.7	369.9	373.4	378.6	383.7
(a)	S.D.	37.7	31.9	36.9	35.4	39.3	39.4	41.3	43.5	43.3	42.4	44.1	44.1	48.3	49.6
3.0% Phe	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3
1.0% pCPA	Mean	393.3	387.7	386.3	391.7	389.3	389.7	391.0	391.3	383.3	393.3	401.0	401.0	400.3	403.0
(b)	S.D.	59.0	60.7	60.8	60.0	63.2	63.6	62.0	59.1	61.5	65.0	69.5	71.0	69.6	76.5
3.5% Phe	N	3	3	3	3	3	3	3	3	3	3	3	3	3	3
1.0% pCPA	Mean	650.0	629.7	623.0	626.3	621.0	619.3	621.0	618.0	622.7	624.3	628.0	629.3	611.7	617.7
(c)	S.D.	92.9	68.5	69.9	77.5	60.6	65.2	64.8	66.5	67.9	79.1	78.9	85.3	87.2	87.4
3.5% Phe	N	5	5	5	5	5	5	5	5	5	5	5	5	5	5
1.0% pCPA	Mean	448.6	430.8	424.0	420.0	411.0	415.2	419.4	416.6	418.8	421.8	425.2	427.4	427.4	435.6
(d)	S.D.	29.5	33.9	29.2	28.2	33.8	33.2	37.9	40.8	41.2	36.3	34.9	33.5	28.7	25.7
3.5% Phe	N	4	4	4	4	4	4	4	4	4		4	4	4	4
1.0% pCPA	Mean	375.5	360.5	347.2	338.8	341.8	344.0	341.5	342.8	348.8		352.0	349.5	356.8	356.0
(e)	S.D.	6.6	14.6	28.6	33.4	28.4	23.4	18.0	21.0	22.1		22.9	22.8	19.7	22.7
3.75% Phe	N	6	6	6	6	6	6	6	6	6	5	5		5	5
1.0% pCPA	Mean	370.8	363.7	366.0	364.0	358.7	358.2	360.0	368.2	370.0	375.4	380.8		382.0	384.4
(f)	S.D.	15.5	14.2	12.5	14.6	14.6	13.3	13.4	17.2	8.6	9.8	9.0		14.2	13.0
4.0% Phe	N	6	6	6	6	6	6	6	6	6	6	6		6	6
1.0% pCPA	Mean	358.8	341.3	341.8	343.7	341.7	343.8	340.8	343.8	349.3	350.2	355.0		361.7	360.3
(g)	S.D.	11.4	14.0	19.1	14.8	13.9	15.9	15.9	17.3	20.0	20.5	20.2		22.9	24.5
4.25% Phe	N	6	6	6	6	6	6	6	6	6	6	6		6	6
1.0% pCPA	Mean	366.8	351.8	342.7	334.3	325.0	322.8	320.7	319.7	313.5	307.5	307.3		307.3	311.7
(h)	S.D.	13.3	14.2	13.5	16.1	19.8	11.6	12.8	14.2	15.8	15.7	15.5		16.7	16.8
	N	6	6	6	5	5	5	5	5	5	4	4	4	4	4
1.0% pCPA	Mean	413.2	422.3	419.3	398.8	399.0	395.0	400.8	401.6	404.6	390.2	400.0	398.0	404.8	406.5
(i)	S.D.	67.3	64.6	67.6	38.7	38.3	42.2	47.5	46.4	47.6	47.3	49.8	53.7	52.7	56.4

Table 27 Series C, Experiment 2

Group	NUMBER OF DAYS ON TEST DIET																	
	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
(a)	7	7	7	7	7	7	7	7	7									
	381.4	385.6	381.3	383.1	382.9	387.3	388.6	391.3	389.6									
	53.9	52.0	47.5	46.4	48.7	49.6	48.2	49.3	53.6									
(b)	3	3	3	3	3	3	3	3	3	3	3							
	404.7	414.7	416.7	418.7	424.7	425.3	430.0	428.7	432.0	428.3	443.0							
	74.6	76.0	81.8	84.2	83.8	79.5	81.7	74.2	73.1	63.9	70.4							
(c)	3	3	3	3	3	3	3	3										
	623.3	618.7	607.7	608.7	597.3	600.3	612.0	608.0										
	86.8	82.3	86.0	85.6	66.9	75.4	89.6	83.8										
(d)	5	5	5	5	5	5	5	5	5	5	5	5						
	433.0	439.8	439.6	443.8	440.8	448.6	449.2	450.2	455.6	456.8	462.6	467.0						
	34.3	28.0	29.5	27.7	30.8	29.8	28.2	32.2	32.4	36.6	33.3	36.0						
(e)	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4		
	361.8	359.8	363.0	370.8	370.8	376.8	377.8	382.0	389.2	389.8	394.0	398.5	388.8	384.8	398.8	406.8		
	25.6	18.1	24.8	23.6	21.6	23.4	26.2	23.9	23.8	20.2	21.6	22.0	23.3	24.1	23.2	23.4		
(f)	5	5	5	5	5	5	5	5	5	5	5	5	5	5		5	5	5
	385.8	391.4	389.6	392.4	401.4	406.6	404.4	409.8	416.2	415.8	418.4	422.4	423.8	429.4		419.0	426.0	431.6
	11.4	14.6	18.0	20.1	20.3	20.9	22.7	22.9	23.4	28.0	25.8	25.5	28.0	29.0		28.9	31.4	40.3
(g)	6	6	6	6	6	6	6	6	6	6	6	6	6	6		6	6	6
	364.5	369.5	373.5	374.2	377.3	383.3	379.8	388.0	392.0	394.0	395.0	396.0	398.2	402.3		408.8	407.7	410.8
	25.5	29.8	26.7	25.1	30.3	30.4	31.7	31.6	34.0	36.5	37.2	39.7	38.6	40.6		43.9	42.9	42.6
(h)	6	6	6	6	6	6	6	6	6	6	6	6	6	6		6	6	6
	313.7	322.7	326.7	327.2	335.5	340.0	338.2	345.7	343.5	347.8	347.7	354.3	353.3	359.0		368.0	370.5	378.7
	15.8	17.7	17.5	23.3	24.9	23.2	23.4	28.4	29.1	33.2	30.8	34.6	34.8	35.8		36.1	35.0	37.8
(i)	4	4	4	4	4	4	4	4	3	3	3	1						
	399.5	403.0	405.2	404.8	407.0	413.0	411.2	415.0	449.3	455.0	454.0	464						
	64.5	66.7	62.2	68.4	74.2	70.0	77.4	79.3	49.7	50.2	54.9							

*Number of animals

Table 28 Series C, Experiment 2 Daily food intake (g/24 hours) as a function of number of days on diet.

DIET (Group)		NUMBER OF DAYS ON TEST DIET													(continued)
		Prediet	1	2	3	4	5	6	7	8	9	10	11	12	13
2.5% Phe 1.0% pCPA (a)	N*	7	7	7	7					7	7	7			
	Mean	26.4	27.3	24.6	26.9					29.4	25.4	31.0			
	S.D.	5.7	6.9	4.7	4.1					3.6	4.8	3.8			
3.0% Phe 1.0% pCPA (b)	N		3	3	3							3	3		
	Mean		13.0	17.7	22.3							36.0	33.0		
	S.D.		7.6	7.6	7.6							2.6	2.0		
3.5% Phe 1.0% pCPA (c)	N		3	3	3	3	3		3	3			3		
	Mean		14.0	25.3	29.0	29.0	34.7		31.7	31.7			31.0		
	S.D.		11.4	7.4	9.6	6.6	24.5		10.7	6.8			14.0		
3.5% Phe 1.0% pCPA (d)	N	5	5	5	5	5			5	5	5	5	5		
	Mean	28.0	9.4	12.8	16.8	17.0			20.6	24.2	24.4	26.8	27.0		
	S.D.	2.9	4.8	5.4	7.1	9.6			6.3	3.8	1.7	3.4	2.6		
3.5% Phe 1.0% pCPA (e)	N	4	4			4	4	4	4	4			4	4	4
	Mean	28.5	2.0			7.5	13.0	18.5	13.5	20.5			24.5	19.5	21.5
	S.D.	1.3	4.0			7.8	3.5	3.4	5.2	3.1			2.4	1.3	1.7
3.75% Phe 1.0% pCPA (f)	N	6	6	6	6			6	6	6	5	5			5
	Mean	26.8	9.2	20.2	17.5			19.8	19.8	22.7	18.6	21.4			24.0
	S.D.	2.1	4.9	2.8	3.5			4.6	3.8	5.8	4.5	7.6			5.7
4.0% Phe 1.0% pCPA (g)	N	6	6	6	6			6	6	6	6	6			6
	Mean	27.0	6.2	12.7	13.2			15.0	12.5	21.8	18.7	21.8			26.8
	S.D.	2.8	4.9	4.1	4.5			4.3	4.4	3.6	5.1	5.7			5.1
4.25% Phe 1.0% pCPA (h)	N	6	6	6	6			6	6	6	6	6			6
	Mean	27.5	6.5	7.2	7.3			7.5	7.7	13.7	9.7	14.3			20.0
	S.D.	1.8	2.7	3.1	2.6			2.1	3.3	3.9	3.2	4.0			5.2
1.0% pCPA (i)	N	3	5	4	4	2	1	1	3	2	1	3	3		1
	Mean	24.7	28.0	29.2	29.8	30.0	23	23	33.3	23.5	23	40.7	32.0		29
	S.D.	11.0	4.3	9.7	9.3	14.1			9.4	12.0		19.3	11.3		

Table 28 Series C, Experiment 2

Group	NUMBER OF DAYS ON TEST DIET																	
	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
(a)	7 30.1 4.6						27.6 1.6											
(b)		3 34.3 3.0		3 36.0 3.0	3 32.3 1.5	3 30.0 3.5	3 35.0 1.7				3 27.3 5.5							
(c)	3 35.0 13.8	3 27.7 13.3			3 24.3 10.7			3 27.3 8.1										
(d)		5 26.8 4.0	5 25.8 3.0	5 24.8 5.4				5 26.2 3.6	5 29.0 5.1	5 28.0 4.8	5 31.0 2.2	5 25.8 4.4						
(e)	4 24.5 4.2	4 25.8 2.8			4 27.5 1.0	4 25.5 1.7	4 27.8 1.5	4 26.8 1.5	4 27.5 0.6			4 28.0 1.8	4 24.8 1.3	4 17.5 4.4	4 25.5 0.6	4 26.2 2.1		
(f)	5 21.2 3.3	5 25.2 4.4	5 22.2 6.4	5 21.0 5.4			5 24.8 4.3	5 22.0 5.3	5 25.2 5.8	5 24.6 4.7	5 24.2 3.3			5 28.0 5.1	5 27.2 5.8	5 23.8 4.2	5 26.2 4.2	5 24.8 5.3
(g)	6 20.7 4.8	6 25.3 5.6	6 24.0 3.2	6 24.0 4.6			6 23.7 6.8	6 25.7 4.3	6 25.3 2.6	6 23.0 2.3	6 24.2 4.4			6 25.2 3.3	6 25.0 3.7	6 26.3 3.6	6 26.2 3.9	6 24.0 3.1
(h)	6 17.8 4.4	6 22.0 6.2	6 22.2 3.6	5 23.2 4.2			6 23.7 4.2	6 22.3 1.5	6 24.7 2.7	6 24.2 4.2	6 22.8 3.1			5 23.6 3.0	6 25.5 2.8	6 24.8 3.4	6 23.5 2.8	6 25.2 2.9
(i)	2 26.5 6.4	2 32.5 5.0		3 35.3 10.2	2 31.0 0	2 20.5 2.1	3 31.3 2.5				3 28.3 11.1	1 32						

* Number of animals

From day 11 on, the animals ate at a rate comparable to prediet levels.

The animals in the third 3.5% Phe 1.0% pCPA diet group, (e), lost nearly 10% of their prediet body weight during the first 3 days of the test diet period (Table 27). By day 4 the animals began to regain the lost weight and continued to grow at a rate of 2.6 g/day. The food consumption of these animals dropped very sharply during the initial days on the test diet and slowly increased to about 20 g/24 hour by day 8 (Table 28). Food intake continued to increase to nearly the prediet level. The lower food intake on day 27 however, may be related to the surgery performed on the animals on day 26 as part of another study (Series C, Experiment 4).

The animals in group (f) lost about 4% of their prediet body weight during the first 5 days of the 3.75% Phe 1.0% pCPA test diet period (Table 27). These guinea pigs then gained weight at a rate of 2.8 g/day until the end of the study. Food intake dropped sharply on day 1 and rose to about 20 g/ 24 hour from days 2 through 11 (Table 28). During the final days of the experiment, daily food consumption approached the prediet rate.

Animals given the 4.0% Phe 1.0% pCPA test diet, group (g), lost about 5% of their prediet body weight during the first day of the study (Table 27). Except for a slight loss on day 6, this weight was maintained until day

7 when the animals began to gain weight. Daily weight gain then averaged 2.8 g/day through the end of the experiment. Food intake dropped during the first day on the diet and rose to about 20 g/24 hour which was maintained through the remainder of the study.

The animals fed the 4.25% Phe 1.0% pCPA test diet had the most severe and sustained weight loss, 16.2% of prediet body weight during the first 10 days of the experiment (Table 27). After day 13 these animals gained weight at a rate of 3.8 g/day until the study was terminated. Food intake dropped on the first day of the study and unlike the other groups, remained at this low level (about 7 g/24 hour) until day 8 (Table 28). Food intake then began to increase and reached about 21 g/24 hour by day 21. Food consumption did not return to prediet levels and fluctuated around 24 g/24 hour until day 31.

Animals in group (i) did not decrease food intake or lose weight when given the 1.0% pCPA diet (Tables 27 and 28). There was a slow but steady increase in mean body weight, despite the fact that one animal lost weight throughout the experiment. This animal subsequently continued to lose weight when given Purina chow and finally had to be destroyed.

In summary, all groups consuming test diets supplemented with Phe lost weight during the initial days on the test diets and then with the exception of group (c), began to steadily gain weight. This was associated with an initial

decline in daily food intake followed by a gradual return to normal, or near normal food consumption. There appeared to be a relationship between the dietary Phe supplement and the day when each group stopped losing weight. Animals in the 1.0% pCPA diet group did not lose weight and the other groups stopped losing weight on the following days: (a) 2.5% Phe 1.0% pCPA 4% sucrose 4% lettuce, at 4 days (Series D, Experiment 1); (b) 3.0% Phe 1.0% pCPA 5% sucrose 5% cabbage, at 3 days; (c) 3.5% Phe 1.0% pCPA 5% sucrose 5% cabbage, at 7 days; (d) 3.5% Phe 1.0% pCPA 7% sucrose 3% cabbage, at 5 days; (e) 3.5% Phe 1.0% pCPA 8% sucrose, at 4 days; (f) 3.75% Phe 1.0% pCPA 8% sucrose, at 6 days; (g) 4.0% Phe 1.0% pCPA 8% sucrose, at 7 days; and (h) 4.25% Phe 1.0% pCPA 8% sucrose, at 11 days. As the magnitude of the Phe supplement increased the duration of the weight loss also tended to increase. In addition, higher dietary sucrose supplements among the 3.5% Phe 1.0% pCPA groups appeared to shorten the duration of weight loss as well as improve daily food intake during the later days of the experiment (Table 28).

Dietary Cabbage: There did not appear to be differences attributable to the presence or absence of cabbage in the diet among the 3 groups eating diets supplemented with 3.5% Phe 1.0% pCPA. Group (e), which did not have cabbage added to the test diet, had elevations of plasma PA and Tyr which were between those of groups (c) and (d); these animals were fed diets supplemented with 5% and 3% cabbage respectively

(Figure 8).

Route of ascorbic acid administration: Animals in groups (a), (b), and (c) received IP ascorbic acid supplements while animals in groups (d) through (h) were given ascorbic acid supplements orally. Except for the possibility of lower PA levels and poor weight gain [group (c)] in animals given IP ascorbic acid (Figure 8, Table 27), no effect could be attributed directly to the route of ascorbic acid supplementation.

On the basis of the data collected in this experiment, 3.5% Phe was concluded to be the optimum dietary Phe supplement. When more than 3.5% Phe was added to test diets, plasma Phe was not significantly increased except when Tyr was also substantially elevated. In addition, food intake and weight gain tended to decline as the dietary Phe supplement increased. Subsequent experiments were undertaken in which 3.5% Phe 1.0% pCPA diets were used to induce stable hyperphe in pregnant and nonpregnant guinea pigs.

Series C, Experiment 3

Introduction

In Series C, Experiment 2 plasma obtained from the saphenous vein and the ophthalmic venous plexus was shown to have nearly equal levels of PA and Tyr. When blood was collected from the severed neck of the animals in Series C, Experiment 1, the plasma levels of PA and Tyr were found to be unexpectedly low in all the test diet groups when compared to the previous 23 days of that study. The markedly lower plasma levels in the severed neck blood from these animals (see below), led to a study in which PA, Phe, and Tyr levels were compared in blood samples obtained from a number of sites, including the severed neck, from the animals in group (e) of Series C, Experiment 2.

Methods

This experiment was performed on the animals in groups (d) and (e) of Series C, Experiment 2 on the final days of that study. All animals were consuming test diets supplemented with 3.5% Phe and 1.0% pCPA and were receiving daily oral ascorbic acid at the time Experiment 3 was done. (see Series C, Experiment 2 for details).

After 25 days on the test diet, 4 animals in group (d) were killed by decapitation between 1340 and 1355 hours,

and blood from the severed neck was collected into heparinized centrifuge tubes . In addition, 4 hours and 10 minutes prior to death, one of these animals was bled from the ophthalmic venous plexus.

The animals in group (e) were bled from both the leg and eye on days 6, 12, 15, 19, and 22. On day 26, these animals were anesthetized with methoxyflurane (CO_2 induction), and blood was collected from the carotid artery, the external or internal jugular vein, and the ophthalmic venous plexus of each animal. Briefly, the surgical procedure was as follows. The ventral aspect of the neck was shaved and cleaned with 70% alcohol. A midline incision was made and the skin reflected laterally. The carotid artery was located and a loop of suture thread placed around the vessel and lifted to expose the artery. The artery was entered with a 26 gauge needle on a heparinized syringe and about 0.75 ml of blood withdrawn. The artery was then tied off with the thread. Blood was then obtained in a similar manner from either the ipsilateral external or internal jugular vein. The skin incision was sutured closed, cleaned, and additional blood collected by the intraorbital method. The entire procedure took between 15 and 25 minutes for each animal and was done between 1015 and 1225 hours. On day 29, these animals were bled from the leg vein, then anesthetized and bled from the ophthalmic plexus, the untied carotid artery, and jugular vein, in the manner described above. Immediately following

the surgical procedure, each animal was killed by decapitation and blood collected from the severed neck. The animals were killed between 1430 and 1645 hours.

All plasma samples were analyzed in duplicate fluorometrically for PA and Tyr and, when sufficient plasma was available, also by paper chromatography for Phe and Tyr. The day 25 sample obtained from the eye in group (d) was also run on the amino acid analyzer, as described above, and is included in Table 26.

Results

Experiment 3 was undertaken to further investigate the unexpectedly low plasma PA and Tyr levels observed in the samples taken from the animals decapitated in group (d). Animals in this group had mean plasma PA levels ranging from 18.3 to 29 mg/100 ml in bloods obtained from the ophthalmic venous plexus on days 8, 11, 17, and 23 (Table 12); however the mean PA level in the 4 samples collected from the severed neck of these animals on day 25 of the study was only 12.0 mg/100 ml (Table 29). When this PA level was compared to the lowest daily mean PA level (day 11) using a paired t-test, the difference was not found to be significant ($t = 1.253$, $df = 3$, two-tailed), although it differed significantly from the day 23 PA level (paired $t = 6.049$, $df = 3$, $p < 0.01$, two-tailed). The single sample collected from the eye 4 hours prior to death had a PA level of 28.4 mg/100,

Table 29 Series C, Experiment 3 Effect of the source of blood on plasma phenylalanines and tyrosine (Mean ±S.D. mg/100 ml), fluorometric and paper chromatographic** analyses.

GROUP			SOURCE OF BLOOD				
Number of Days on diet 3.5% Phe 1.0% pCPA			Severed Neck	Eye	Leg	Arterial	Venous
(d) 25 days	Fluoro.	N***	4	1			
		PA	12.05±3.44	28.4			
		Tyr	2.52±1.33	7.4			
	Paper	N	4	1			
		Phe	3.2±2.5	20.0			
		Tyr	1.8±1.5	12.0			
(e) 26 days	Fluoro.	N		4		4	4
		PA		17.80±7.69		18.28±6.10	16.60±5.64
		Tyr		4.15±1.79		4.45±1.45	3.12±1.17
	Paper	N		4		4	4
		Phe		8.0±7.1		9.5±9.0	9.0±8.0
		Tyr		5.0±2.3		5.5±1.9	5.0±2.3
(e) 29 days	Fluoro.	N	4	4	4*	4+	4
		PA	7.22±5.89	16.48±7.84	12.1	11.97±5.16	10.38±2.89
		Tyr	0.98±0.76	2.48±1.26	2.40±1.18	1.50±1.01	1.38±0.38
	Paper	N	4	4		2	3
		Phe	5.6±2.1	6.8±7.5		6.5±4.9	3.0±0
		Tyr	6.0±4.24	7.5±7.1		5.5±4.9	3.0±1.7

* only one sample analyzed for PA
** For calculation, paper chromatographic values of <6, <5, <4, <3, <2, were taken as 3, 3, 2, 2; 1, mg/100 ml respectively.
*** number of samples
\+ one sample not analyzed for PA

in contrast to the level of 10.1 mg/100 ml in the neck blood from the same animal; Tyr levels also showed a similar disparity, 7.4 vs 2.0 mg/100 ml (Table 29). Fluorometric Tyr levels did not differ significantly from the day 11 levels (paired $t = 2.898$, $df = 3$, two-tailed), but did differ marginally from the day 23 levels (paired $t = 4.107$, $df = 3$, $p < .05$, two-tailed). With both PA and Tyr levels the trend was clearly toward lower plasma concentrations in the neck samples. This trend was also apparant among the other groups in Series C, Experiment 1.

The paper chromatographic estimations of plasma Phe and Tyr in the bloods collected from the neck of the animals in group (d) were also lower than the levels observed in blood obtained from the ophthalamic venous plexus (compare Tables 13 and 29). In addition, the paper estimation of Phe and Tyr in the day 25 eye sample (Table 29) is considerably greater than the day 25 neck sample from the same animal (Phe < 3, Tyr < 2 mg/100 ml).

Animals in group (e) were bled from a number of different sources while consuming a diet supplemented with 3.5% Phe and 1.0% pCPA. As discussed above (Series C, Experiment 2), there were no statistically significant differences between plasma obtained from the ophthalmic venous plexus and the saphenous vein with respect to either PA or Tyr levels on days 6, 12, 15, 19, or 22 of the study (Table 19).

On day 26, the animals in group (e) were bled from the ophthalmic venous plexus, the carotid artery, and the jugular vein; the mean plasma PA, Phe, and Tyr levels are included in Table 29. The fluorometric and paper chromatographic measurements of PA, Phe, and Tyr in the arterial and venous samples were compared statistically to the ophthalmic level by paired t-tests. None of the levels differed significantly at the 0.05 level on day 26.

On day 29, these animals were again bled from the eye, carotid artery, and the jugular vein, and in addition, from the saphenous vein and from the severed neck following decapitation (Table 29). The amino acid data from each source were compared to the eye blood levels by paired t-tests. Fluorometric measures of PA and Tyr in the neck bloods differed significantly from the eye blood levels (PA: $t = 5.83$, $df = 3$, $p < .01$, two-tailed; Tyr: $t = 6.040$, $df = 3$, $p < .01$, two-tailed), although neither the paper estimates of Phe or Tyr differed significantly between eye and neck samples. The only other significant difference among the day 29 samples was between the eye and arterial fluorometric Tyr levels ($t = 5.426$, $df = 3$, $p < .02$, two-tailed). It was not possible to fluorometrically assay PA in 3 of the 4 samples obtained from the leg vein on day 29; however the results of Series C, Experiment 2 demonstrated that PA and Tyr levels were indeed equal in samples from the leg vein and the eye on days 6, 12, 15, 19 and 22 of the study.

It was concluded that levels of Phe, PA, and Tyr in blood obtained from the saphenous vein are a reliable indicator of these amino acids in the guinea pig. There was a definite trend toward lower levels in blood from the severed neck and this method of blood collection was therefore not used in subsequent studies. No anesthesia is required for blood collection by the leg method, a distinct advantage over the ocular technique. The saphenous vein was therefore used for blood sampling in subsequent studies.

Series C, Experiment 4

Introduction

The results of Series C, Experiments 1 and 2 indicated that adult female guinea pigs fed diets supplemented with 3.5% Phe and 1.0% pCPA are hyperphenylalaninemic and have only moderate plasma Tyr elevations. The elevations of plasma Phe were often above 15 mg/100 ml, the level associated with significant risk to the human fetus (Tables 4, 6). This hyperphe was shown to be present for at least 30 days. In these experiments blood generally was collected between 0900 and 1230 hours. It was therefore of importance to determine if the plasma PA, Phe, and Tyr concentrations of blood taken during the morning was representative of levels during the remainder of the day. In Series C, Experiment 4, animals consuming a 3.5% Phe 1.0% pCPA diet were bled every 4 hours during a 12 hour period and plasma amino acids determined.

Methods

At the conclusion of Series C, Experiment 2, the 5 animals (105 days of age) in group (g) were switched from the 4.0% Phe 1.0% pCPA diet to test diets supplemented with 3.5% Phe 1.0% pCPA and 8% sucrose. As a part of another study, 3 of these animals also had 2% cabbage added to diet.

The animals received twice daily oral ascorbic acid supplements of 25 mg/300 g. The animals were bled from the saphenous vein at approximately 1130 hours, 4, 9, 12 and 16 days after being given the 3.5% Phe 1.0% pCPA diet. On day 25 the animals were bled 4 times during a 12 hour interval; blood was taken from each animal at approximately 0930, 1330, 1725, and 2120 hours. At each of these times less then 20 minutes was required to bleed all 5 animals. Heparinized plasma was used for duplicate fluorometric PA and spectrophotometric Tyr determinations. When sufficient plasma was available, Phe and Tyr were also estimated by paper chromatography.

Animals were examined and weighed daily and food intake determined in g/24 hours. When fresh urine was available, $FeCl_3$ tests for phenylketones were performed.

Results

Table 30 summarizes the plasma PA, Phe, and Tyr data during the 25 days of this study; the prediet values were obtained prior to Series C, Experiment 2. Mean daily plasma PA levels ranged from 19.7 to 29.6 mg/100 ml and are comparable to those of previous groups fed 3.5% Phe 1.0% pCPA diets (Cf Tables 12, 19, 20). Paper chromatographic estimations of plasma Phe were done on most of the samples and showed that mean plasma Phe was elevated and in the range of 15 to 20 mg/100 ml (Table 30). These Phe levels are similar

Table 30 Series C, Experiment 4 Mean (±S.D.) phenylalanines and tyrosine as a function of the number of days on diet, 3.5% Phe 1.0% pCPA 8% sucrose, (mg/100 ml).

		NUMBER OF DAYS ON DIET					
		Prediet	4	9	12	16	25+
Fluorometric (Spectrophotometric)	N*	5	5	5	5	5	5
	PA	1.40±0.16	19.70 ±5.48	23.64 ±10.73	39.58 ±9.46	34.88 ±14.43	22.92 ±14.34
	Tyr	1.16±0.22		2.10 ±0.76	7.20 ±4.99	6.42 ±6.21	3.66 ±1.79
	P/T**	1.21		11.26	5.50	5.43	6.26
Paper Chromatographic	N	1	2	1	5	4	5
	Phe	<6	12.0 ±8.5	>32	16.8 ±5.9	20.0 ±10.3	16.0 ±12.2
	Tyr	<4	2.0±0	<4	5.8±3.7	7.5±7.1	3.4±1.9
	P/T		6.0		2.9	2.7	4.7
Food Intake	N	5	5	5	5	5	5
	g/24h	26.2±1.5	28.2 ±4.5	22.6 ±3.2	29.4 ±4.9	30.2 ±4.1	28.8 ±5.3

* Number + 0930 hours samples.
** Phenylalanine(s): Tyrosine ratio.

to those of other groups consuming 3.5% Phe 1.0% pCPA diets (cf Tables 13, 24, 26). Mean plasma Tyr levels ranged from 2.1 to 7.5 mg/100 ml (spectrophotometric and paper estimations, Table 30); the mean Tyr levels on days 12 and 16 are slightly higher than those observed in earlier studies (cf Tables 12, 13, 19, 20, 24, 26). One animal had Tyr concentrations of between 12 and 18 mg/100 ml on days 12 and 16, accounting for the higher mean levels on these days. Three (3) $FeCl_3$ tests were done and all were positive.

Table 30 also includes the food intake in g/24 hours on each of the days the animals were bled. The animals consumed the test diet at a rate comparable to their prediet (Purina chow) intake. These animals had previous experience with Phe supplemented diets and therefore did not exhibit the initial decline in food intake characteristic of test diet-naive animals. The near-normal food consumption was reflected in the weight gain of the animals. Every animal gained weight during the 25 days of the study and the average increase was 30.2 g.

Figure 9 illustrates the mean plasma PA and Tyr (spectrophotometric) levels during a 12 hour interval on day 25 of the study. It is clear that the morning (0930 hours) PA level was the lowest during the period studied and that PA continued to rise until at least 2120 hours. Mean PA reached 50.7 mg/100 ml at 2120 hours. Figure 10 includes the paper chromatographic estimates of plasma

Figure 9. Series C, Experiment 4. Mean (±S.D.) plasma total phenylalanines (fluorometric method) and tyrosine (spectrophotometric method) during a 12 hour period. Solid line illustrates phenylalanine data and dashed line the tyrosine data.

Figure 10. Series C, Experiment 4. Mean (±S.D.) plasma phenylalanine (solid line) and tyrosine (dashed line) during a 12 hour interval as determined by paper chromatography.

Figure 11. Series C, Experiment 4. Mean (±S.D.) plasma phenylalanines (solid line) and tyrosine (dashed line) during a 12 hour period in a group of animals fed a 3.75% Phe 1.0% pCPA test diet. Fluorometric method.

Figure 9

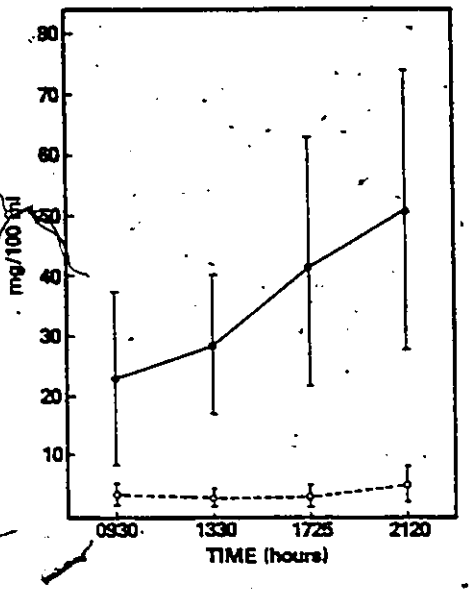


Figure 10

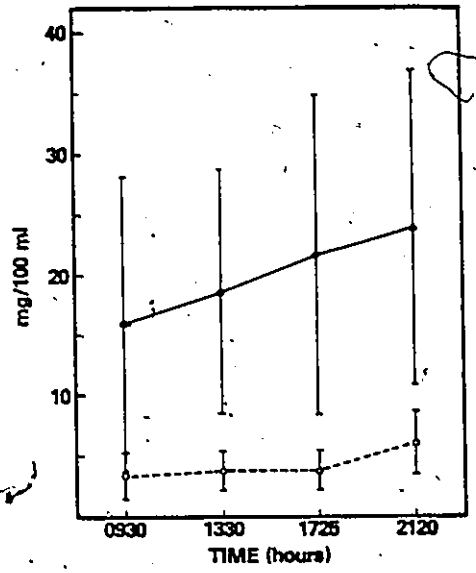
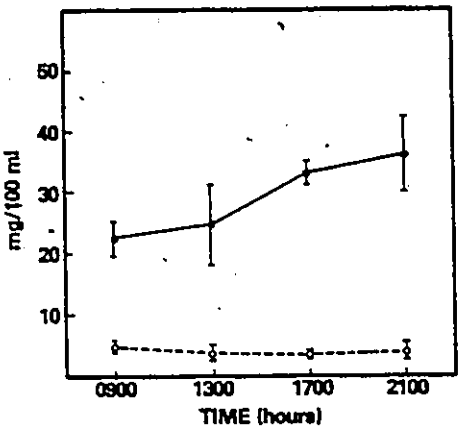


Figure 11



Phe and Tyr during the same interval and indicates that plasma Phe also rose from 0930 to 2120 hours. Mean plasma Phe was 23.8 mg/100 ml at 2120 hours. In sharp contrast to PA and Phe, plasma Tyr levels remained quite stable and increased only slightly from 1725 to 2120 hours (Figures 9 and 10).

An identical study was also undertaken with 3 animals which had been consuming test diets supplemented with 3.75% Phe 1.0% pCPA and 8% sucrose for 31 days. Plasma PA was found to increase from 0900 hours to 2100 hours while Tyr levels changed very little (Figure 11). The pattern was similar to that observed in the animals fed the 3.5% Phe 1.0% pCPA diet (cf Figure 10).

Series C, Experiment 5

Introduction

Experiments 1 and 2 of Series C demonstrated that the daily food intake of guinea pigs was decreased when their diets were supplemented with Phe and pCPA. This poor food intake was most severe during the first few days after the supplemented test diets were presented to the animals. Dietary supplements of up to 8% sucrose did not entirely prevent this poor food intake. Work in this laboratory has shown that poor maternal nutrition is a factor contributing to pregnancy loss in guinea pigs (Series E, Experiment 2). Studies were therefore undertaken to improve dietary intake of Phe and pCPA supplemented guinea pig diets.

Aspartame [L-aspartyl-L-phenylalanine methylester, (Asp)] is a synthetic super sweetener which is 180 to 200 times sweeter than sucrose (American College of Nutrition 1974). An important advantage of Asp, which is a consequence of its intense sweetness, is that relatively small quantities are needed to sweeten diets and therefore significant dilution of dietary nutrients is not a problem. Aspartame was therefore added to 3.5% Phe 1.0% pCPA test diets at concentrations equivalent to the sweetness of about a 15 to 35% sucrose supplement (ie. 0.075 to 0.175% Asp). These Asp

sweetened diets were fed to guinea pigs and daily food intake, body weight and plasma PA, Phe, and Tyr monitored. In addition, hepatic Phe hydroxylase activity was determined in some of these animals.

Methods

All animals used in this experiment were Hartley strain guinea pigs obtained from M. & L. Farms. There were six experimental groups in which animals were given test diets supplemented with 3.5% Phe 1.0% pCPA and either 8% sucrose or varying amounts of Aspartame. Baseline food intake (g/24 hours) and plasma Phe and Tyr data were obtained when the animals consumed either Purina chow or a laboratory prepared diet supplemented only with a low level of Aspartame (0.025%). All blood samples were taken between 0900 and 1350 hours by the leg method previously described. During both the prediet and test diet time periods animals were examined and weighed daily and food intake was determined on a g/24 hours basis. Except when consuming Purina chow the animals were given daily oral ascorbic acid (50 mg/300 g body weight). $FeCl_3$ tests for phenylketones were done on fresh urine when animals voided while being bled or weighed. Plasma Phe, PA, and Tyr were determined in duplicate by the fluorometric methods. When sufficient plasma was available Phe and Tyr were also determined by paper chromatography.

At the conclusion of the experiment a number of animals were bled, killed by decapitation, and their livers quickly removed, weighed, and frozen on solid CO_2. Twenty four (24) hours later the frozen liver samples were homogenized in 0.1M phosphate buffer and assayed for Phe hydroxylase activity.

A total of 6 experimental groups and one control group were included in the experiment:

Group (a) (8% sucrose). Six (6) animals, 89 days of age, were given a diet supplemented with 3.5% Phe 1.0% pCPA 8% sucrose. Prior to this experiment, these animals had consumed only Purina chow. One animal refused to eat the test diet and was returned to Purina chow and is included in Group (g) (below). The remaining animals were bled approximately every 3 to 4 days and continued on the test diet for 33 days, at which time 2 were killed for Phe hydroxylase assay.

Group (b) (0.075% Aspartame). Five (5) animals, 87 days of age, were given a diet supplemented with 3.5% Phe 1.0% pCPA 0.075% Aspartame. For 3 days before receiving this test diet the animals consumed a 0.025% Aspartame diet; previous to this time, these animals comprised Group (e) (see below). Blood was collected after 3 and 6 days on the test diet and the animals were then killed for Phe hydroxylase determination.

Group (c) (0.10% Aspartame): Initially 5 animals

were included in this group but 2 were subsequently found to have abnormally elevated baseline Tyr levels and were therefore excluded (see Appendix B). The 3 animals, 76 days of age, were given a test diet supplemented with 3.5% Phe 1.0% pCPA 0.10% Aspartame for a period of 8 days. Prior to receiving the test diet these animals consumed only an 0.025% Aspartame diet. During the test diet period the guinea pigs were bled after 5 and 8 days and were then returned to the 0.025% Aspartame diet for 3 days to allow food intake to return to normal levels. These animals were then used in group (f) (see below).

Group (d) (0.125% Aspartame). Six (6) animals were originally assigned to this group however, one was found to have an elevated baseline plasma Tyr level and was therefore excluded (see Appendix B). The five (5) remaining guinea pigs, when 89 days of age, were given the 3.5% Phe 1.0% pCPA 0.125% Aspartame test diet after previously eating only Purina chow. These animals were given this test diet for up to 33 days and were bled approximately every 3 to 4 days during this period. One animal refused the test diet and was returned to Purina chow and is included in group (g) (see below).

Group (e) (0.150% Aspartame). Five (5) animals, 76 days of age, were given the 3.5% Phe 1.0% pCPA 0.150% Aspartame test diet after previously consuming only a 0.025% Aspartame diet. Animals were bled after 5 and 8 days on the test diet and were then returned to the 0.025% Asp diet

for three days. These animals were then given the 3.5% Phe 1.0% pCPA 0.075% Aspartame diet, forming Group (b) described above.

Group (f) (0.175% Aspartame). After 3 days consuming a 0.025% Aspartame diet, the 3 animals previously used in Group (c) (above) were given a 3.5% Phe 1.0% pCPA 0.175% Aspartame test diet. These animals were 87 days of age at this time. The animals were bled after 3 and 6 days on the test diet and then killed for Phe hydroxylase determination.

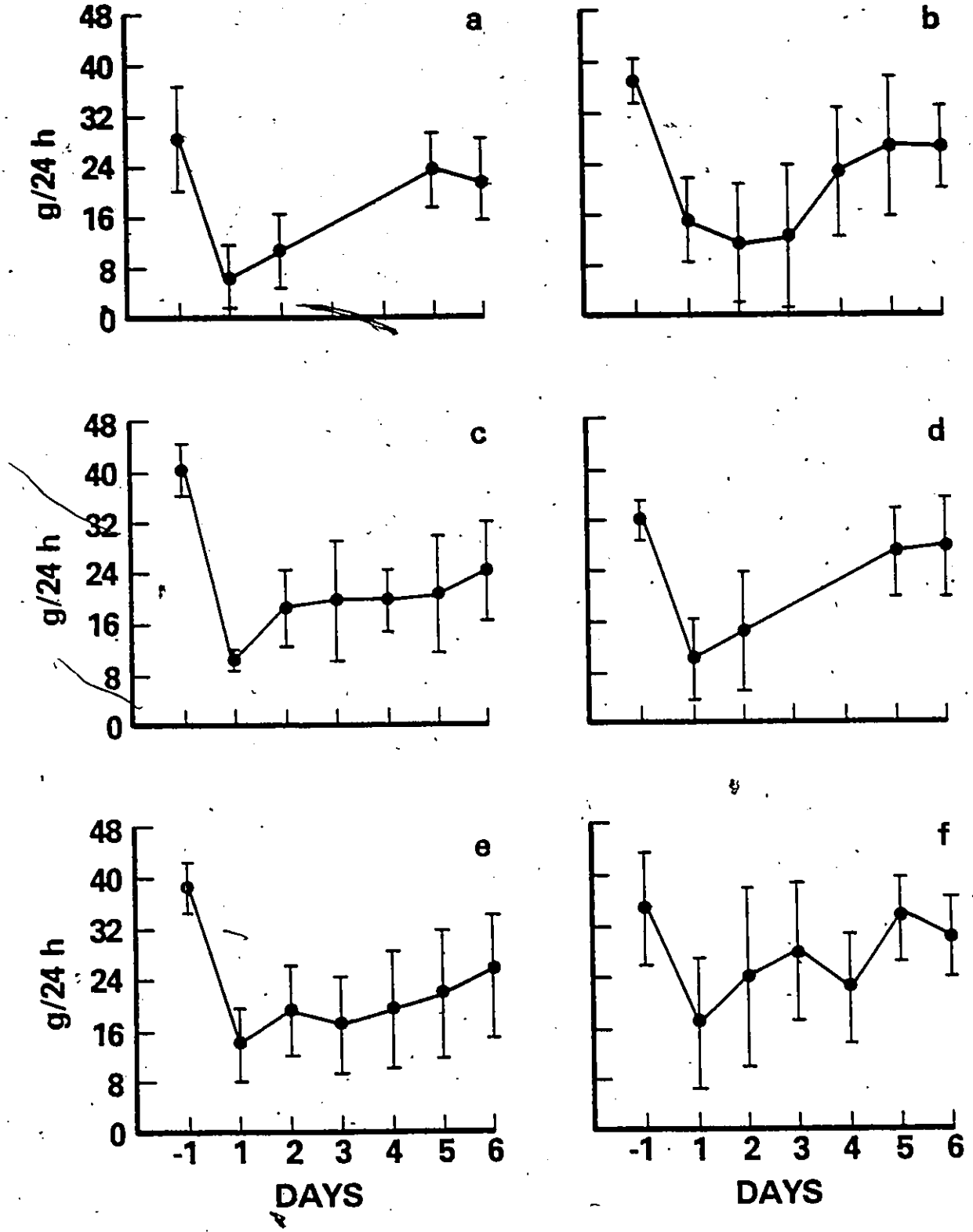
Group (g) (Purina control). Six (6) guinea pigs were included in this group to provide normal tissue for hepatic Phe hydroxylase assay. All were consuming Purina chow when killed and none had ever consumed more than 2g of Phe or pCPA supplemented diets; only the animals removed from groups (a) and (d) had access to such diets. At the time of death, 3 animals were 166 days of age and 3 were 123 days of age. Plasma for Phe and Tyr assay was collected from each animal in this group.

Results

Food Intake: The daily food intake of the animals eating the 6 test diets was determined in g consumed per 24 hours (g/24 hours). Figure 12 illustrates these data for the first 6 days of the 3.5% Phe 1.0% pCPA test diet period and for the day immediately preceding the test diet

Figure 12. Series C, Experiment 5. Mean (±S.D.) daily food intake for the first six test diet days and the day prior to the test diet period. Test diet group is indicated in upper right of each panel.

Figure 12



period. It is clear that in all diet groups food intake decreased dramatically during the first days on the supplemented diets. One animal in each of groups (a) and (d) continued to consume such low quantities of test diet (less than 1g per day average) that both had to be returned to Purina chow on day 5 of the study. Analysis of variance of the daily food intake data indicated significant effects for both the number of days of the study ($F = 24.239$, $df = 6, 37$ $p < .0001$) and for type of diet ($F = 3.952$, $df = 5, 37$ $p = .0023$). Multiple comparisons using the Student-Newman-Keuls procedure indicated that only the prediet (day -1, Figure 12) food intake differed significantly ($p < .05$) from all other days. In order to compare the effect of the sweeteners on test diet intake independently of the prediet intake, the data from the 6 test diet days were analyzed separately (without the prediet intake data) by ANOVA. This analysis also revealed significant effects for both the number of days on the test diets ($F = 10.990$, $df = 5, 31$ $p < .001$) and for the composition of the diet ($F = 3.152$, $df = 5, 31$ $p = 0.0109$). Similar ANOVA using repeated measures was also performed, however this method required that some of the data be excluded. Test diet days 3 and 4 were excluded because some animals did not have food consumption measured on these days. The two animals which were dropped from the study on day 5 because of inadequate food intake were also excluded from this ANOVA. This ANOVA

also revealed a highly significant effect for the day of the study both with and without the prediet food intake (prediet included: $F = 66.215$, $df = 4, 20$ $p < .0001$; without prediet; $F = 52.621$, $df = 3, 19$ $p < .0001$). The repeated measures ANOVA however, failed to detect a significant effect for the test diet composition ($F = 0.806$, $df = 5, 19$). When the mean intakes for each of the first 6 days of the test diet period (across all diets) were compared using the Student-Newman-Keuls multiple range test it was found that three significantly different ($p < .05$) subsets of days existed: days 1 and 2, days 2, 3, and 4, and days 4, 5 and 6 (in order of increasing intake). These subset groupings are consistent with the observation that dietary intake increased daily from days 1 to 6. Multiple comparisons of the mean food intake data for each diet (pooled across days) revealed two subgroups of diets which differed significantly ($p < .05$) in overall food intake. Only two diet groups, (a) 8% sucrose and (f) 0.175% Aspartame, were not included in both subsets and therefore differed significantly from one another in overall food intake. Although this analysis suggests a significant difference between groups (a) and (f), when ANOVA was performed using the mean intake of each animal during the test diet period, a significant difference between test diet groups could be not detected. The mean intake for group (a) was 13.9 g/24 hour and for group (f) the mean was 25.7 g/24 hour; all other groups had inter-

mediate food consumption rates. Although the 0.175% Aspartame diet did not prevent a significant decline in food intake when the animals were initially presented with the test diet (Figure 12), it was associated with the highest average food intake among all the test diet groups. In addition, it was only in the 0.175% Asp group that the difference in food intake compared to the 8% sucrose animals approached statistical significance.

Body Weight: The mean daily body weight of the animals in each experimental group is illustrated in Figure 13 for both the test diet period and the 3 days immediately proceeding this period. There was considerable variation in the body weights within each group, as indicated by the standard deviations shown in Table 31. In this figure (Figure 13) the dashed line refers to the mean weight of all the animals in groups (a) and (d), including the guinea pigs refusing the diet which were deleted from the study after 4 days. Groups (a), (c), and (e) lost weight during the first 6 days of the study while groups (b), (d), and (f) maintained their prediet body weights more effectively, although when all the animals in group (d) were considered, this group also lost weight (Figure 13, Table 31). Steady weight gain during the first 6 days was not observed in any test diet group. Animals in groups (a) and (d) however, remained on the test diets for over 30 days and during this period both slowly gained weight, surpassing their pretest diet weights

Figure 13. Series C, Experiment 5. Mean daily body weight during the test diet period and the preceeding three days of each group. Test diet group indicated in upper right of panel. Dashed curves include animals which refused the test diet and were subsequently deleted from the study.

Figure 13

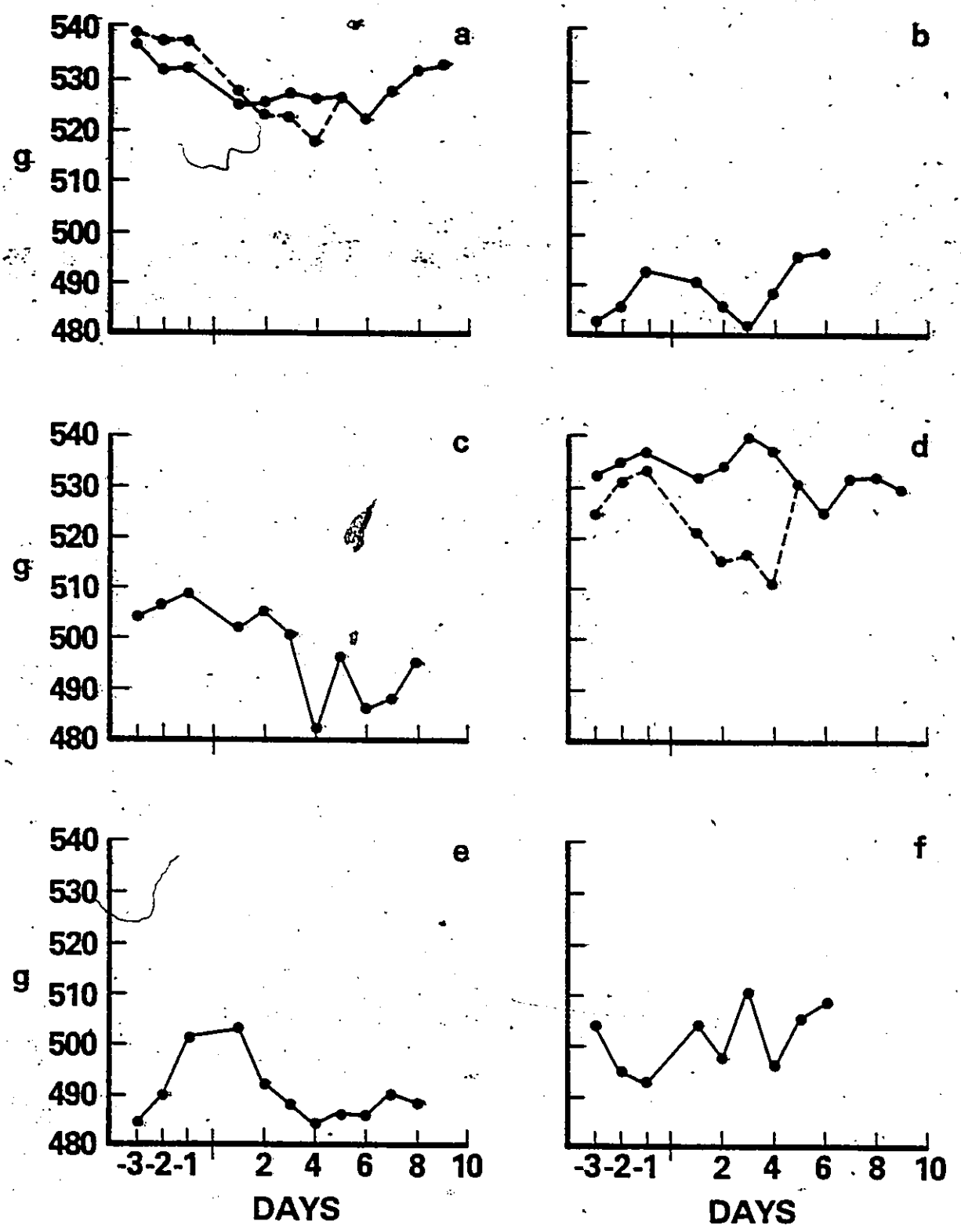


Table 31 Series C, Experiment 5.

Mean (±S.D.) daily body weight during the test diet period (days 1 to 9) and the three days prior to the test diet period (days -1 to -3).

(GROUP) SWEETENER	NUMBER OF DAYS ON TEST DIET (3.5% Phe 1.0% pCPA + sweetener)											
	-3	-2	-1	1	2	3	4	5	6	7	8	9
(a) 8% sucrose	6* 539.2 ±36.0	6 537.8 ±31.2	6 538.0 ±28.8	6 528.2 ±30.5	6 523.0 ±27.9	6 522.7 ±33.6	6 517.7 ±38.4	5 526.8 ±39.0 **	5 522.0 ±41.2 **	5 528.0 ±39.8 **	5 532.2 ±37.4 **	5 532.8 ±41.5 **
(b) 0.075% Asp	5 482.8 ±30.2	5 485.6 ±42.9	5 492.6 ±44.2	5 490.2 ±44.4	5 485.8 ±52.6	5 481.4 ±56.0	5 488.4 ±50.3	5 495.6 ±46.6	5 496.6 ±45.9			
(c) 0.10% Asp	3 504.3 ±17.6	3 506.7 ±19.7	3 508.7 ±6.7	3 502.0 ±2.0	3 505.3 ±9.5	3 500.7 ±18.0	3 481.3 ±19.7	3 496.3 ±19.8	3 486.0 ±19.1	3 488.0 ±14.9	3 495.3 ±12.7	
(d) 0.125% Asp	5 525.0 ±36.2	5 531.0 ±21.5	5 533.4 ±32.7	5 521.2 ±37.4	5 515.2 ±49.7	5 517.0 ±57.7	5 510.8 ±61.2	4 530.8 ±14.7 **	4 525.2 ±16.7 **	4 532.0 ±23.1 **	4 532.2 ±18.5 **	4 529.8 ±20.8 **
(e) 0.150% Asp	5 484.4 ±24.5	5 490.0 ±21.3	5 501.6 ±20.7	5 503.2 ±27.7	5 492.4 ±27.5	5 488.4 ±30.7	5 484.2 ±32.2	5 486.6 ±26.4	5 486.0 ±31.8	5 490.2 ±36.5	5 488.8 ±32.0	
(f) 0.175% Asp	3 504.0 ±17.1	3 495.3 ±18.1	3 492.7 ±8.6	3 504.3 ±2.9	3 497.7 ±6.8	3 510.7 ±9.1	3 496.0 ±14.5	3 506.0 ±13.5	3 509.0 ±13.9			

Continued...

Table 31 Series C, Experiment 5
(Con't.)

(GROUP) SWEETENER	NUMBER OF DAYS ON TEST DIET (3.5% Phe 1.0% pCPA + sweetener)											
	-3	-2	-1	1	2	3	4	5	6	7	8	9
(a)** 8% sucrose	5 537.2 ±39.9	5 531.8 ±30.8	5 532.2 ±28.0	5 525.0 ±33.0	5 525.4 ±30.5	5 527.4 ±35.3	5 526.2 ±36.0					
(d)** 0.125% Asp	4 532.2 ±37.4	4 535.0 ±22.6	4 537.2 ±36.4	4 532.0 ±32.9	4 534.0 ±30.6	4 540.5 ±27.6	4 537.5 ±15.4					

* Number of animals.

** excluding animal not eating which was deleted from from the study after day 4.

by 10 g in the 8% sucrose group and by 20 g in the 0.125% Asp group.

Plasma Phe, PA, and Tyr: Plasma phenylalanines were measured both by fluorometry and by paper chromatography. Table 32 summarizes these data for plasma collected prior to and throughout this experiment; as noted in the table, levels of Phe not detectable by paper chromatography (< 6 mg/100 ml) were taken as 3 mg/100 ml for purposes of calculation and analysis. Table 32 indicates that the extent of plasma PA and Phe elevation is comparable to that observed in previous experiments with 3.5% Phe 1.0% pCPA supplemented diets (cf Tables 12, 13, 18, 19, 24). In addition, groups (a), 8% sucrose, and (d), 0.125% Aspartame, were continued on their respective diets for over 30 days and maintained their elevated PA and Phe levels throughout this period (Table 32). The fluorometric and chromatographic data were analysed by ANOVA following logarithmic transformation. For this analysis these data were grouped into 3 time intervals corresponding to prediet (day 0), days 2 - 5, and days 6 - 8, so that all test diet groups were represented by their first two test diet plasma samples and their prediet baseline Phe levels. ANOVA of the fluorometric PA data revealed a significant effect for the day of the plasma sample ($F = 154.935$, $df = 2, 18$ $p < .0001$), but no significant effect for the type of diet ($F = 1.479$, $df = 6, 18$). Similar analysis of the PA data from only the test diet period, ie. excluding prediet

Table 32 Series C, Experiment 5. Plasma total phenylalanines (PA) and phenylalanine (Phe), mean ±S.D. (mg/100 ml), as a function of the number of days on 3.5% Phe 1.0% pCPA test diets: Effect of sweeteners.

GROUP		PREDIET	NUMBER OF DAYS ON TEST DIET									
SWEETENER	*	0	2-5	6-8	9	13	16	19	21	23	26	30-33
	N	5	5**	5	5	5	5	5	5	5	5	5
(a)	F	1.16 ±0.15	9.94 ±5.34	12.28 ±6.48	23.90 ±6.36	16.20 ±4.35	15.48 ±5.88	7.86 ±2.17	17.30 ±9.62	20.00 ±3.55	16.38 ±4.00	11.68 ±5.49
8% sucrose	P	3.0±0	4.2± 2.5	11.0±6.1	11.6± 7.1	6.6±2.6	7.8±4.0	5.2±3.0	7.2±5.4	6.2±2.9	5.8±3.0	8.0±5.0
	%		42.3%	89.6%	48.5%	40.7%	50.4%	66.2%	41.6%	31.0%	35.4%	68.5%
	N	5**	5	5								
(b)	F	1.22 ±0.19	24.32 ±14.38	22.56 ±7.37								
0.075% Asp	P	3.0±0	9.0± 6.8	13.0±6.8								
	%		37.0%	57.6%								
	N	3	3	3								
(c)	F	1.87 ±0.15	14.17 ±6.01	11.83 ±6.07								
0.10% Asp	P	3.0±0	4.0± 1.7	7.3±7.5								
	%		28.2%	61.7%								
	N	4	4	4	4	4	4	4	4	4	4	3
(d)	F	1.12 ±0.15	17.42 ±11.71	16.75 ±9.73	19.80 ±10.34	21.90 ±14.23	23.10 ±7.06	11.32 ±6.26	20.32 ±8.03	21.50 ±10.28	18.30 ±12.45	16.23 ±4.72
0.125% Asp	P	3.0±0	9.0± 9.0	15.2± 10.5	10.2±8.2	13.2± 12.8	14.0±7.1	12.8±7.1	10.2±5.4	9.0±7.0	9.5±7.7	8.3 ±6.9
	%		51.7%	90.7%	51.5%	60.3%	60.6%	113.1%	50.2%	41.9%	51.9%	51.3%

Continued...

Table 32 (Con't) Series C, Experiment 5

GROUP		PREDIET	NUMBER OF DAYS ON TEST DIET								
SWEETENER	*	0	2-5	6-8	9	13	16	19	21	23	26	30-33
	N	5	5	5								
(e)	F	1.46 ±0.18	16.68 ±6.62	17.28 ±13.52								
0.150% Asp	P	3.0±0	7.8 ±4.5	11.8± 12.2								
	%		46.8%	68.3%								
	N	3	3	3								
(f)	F	1.53 ±0.35	21.83 ±6.24	14.67 ±6.15								
0.175% Asp	P	3.0±0	12.0 ±7.2	5.3±4.0								
	%		55.0%	36.1%								
	N	6**										
(g)	F	1.18 ±0.24										
Purina	P	3.0±0										
	%											

* N = number of samples
F = fluorometric determination (PA)
P = paper chromatographic determination (Phe); values of < 6 taken as 3 mg/100 ml for calculation purposes.
% = (P/F) 100
** one sample not analyzed by chromatography

plasma levels, did not reveal significant effects for either days ($F = 0.001$, $df = 1, 11$) or diets ($F = 1.443$, $df = 5, 11$). The highly significant effect for days in the first ANOVA was due to the difference between the prediet and test diet plasma PA levels, and not to differences in PA levels during the test diet interval. The test diet PA data were also analyzed by ANOVA with repeated measures and again there were no significant effects for either the number of days on the test diet ($F = 0.584$, $df = 1, 19$) or the diet composition ($F = 1.324$, $df = 5, 19$).

The paper chromatographic plasma Phe data were also transformed and subjected to ANOVA. The results are comparable to the analyses of the PA data, in that ANOVA of data Phe from the three intervals revealed a significant effect for days ($F = 15.179$, $df = 2, 18$ $p < .0001$); this significance disappeared when the prediet Phe levels were excluded ($F = 1.573$, $df = 1, 11$). There were no significant effects for the diet composition in either analysis (all 3 intervals: $F = 0.628$, $df = 6, 18$; test diet only: $F = 0.697$, $df = 5, 11$). ANOVA with repeated measures of the transformed Phe data from the test diet period also did not reveal significant effects for the number of days or diet composition (days: $F = 1.199$, $df = 1, 17$; diets: $F = 0.580$, $df = 5, 17$). It is clear that both PA and Phe were significantly elevated during the 3.5% Phe 1.0% pCPA test diet period. In addition, the elevated plasma PA and Phe levels did not differ signi-

ficantly from days 2 through 8 nor among the various sweetener diet groups.

The percentage of PA accounted for by Phe during the test diet period (see Table 32) varied from 24.6% to 113.1% and was often less than the percentage in previous experiments. (cf Tables 13, 24, 30).

Plasma Tyr levels were also measured fluorometrically and chromatographically and are summarized in Table 33. This table indicates that all test diet groups had moderate elevations of plasma Tyr as measured by both methods. In most cases the levels were in the range observed in previous experiments with 3.5% Phe 1.0% pCPA diets (cf Tables 12, 13, 18, 19, 24), although on a number of occasions the mean level was greater than 6 mg/100 ml. As in previous experiments, fluorometric and chromatographic determinations of plasma Tyr agreed well.

Ferric Chloride Tests: Fresh urine was tested for the presence of phenylketones with $FeCl_3$. All tests done while animals were consuming the test diets were positive and the following number were performed in each group; (a) 6, (b) 1, (c) 6, (d) 0, (e) 2, and (f) 8. As observed previously, the positive green colour usually faded within 2-3 minutes.

Phenylalenine Hydroxylase Activity: Phe hydroxylase activity, expressed as μMoles of Tyr produced per hour per g wet weight of liver tissue, was estimated in a number of

Table 33 Series C, Experiment 5. Plasma tyrosine (mg/100 ml, mean ±S.D.) as a function of the number of days on 3.5% Phe 1.0% pCPA test diets: effect of sweeteners.

GROUP		PREDIET	NUMBER OF DAYS ON TEST DIET									
SWEETENER	*	0	2-5	6-8	9	13	16	19	21	23	26	30-33
	N	5	5	5	5	5	5	5	5	5	5	5
(a)	F	1.26± 0.40	2.38± 0.83	6.54± 5.72	5.18± 0.50	4.28± 1.70	3.94± 2.94	4.04± 3.05	4.74± 2.85	4.26± 1.63	3.48± 1.41	3.06±1.40
8% sucrose	P	2.0±0	2.0±0	5.2±7.2	3.0±1.4	2.6±1.3	3.6±3.6	3.0±2.2	3.0±2.2	2.9±1.2	3.0±1.4	2.6±1.3
	%	158.7%	84.0%	79.5%	57.9%	60.7%	91.4%	74.3%	63.3%	68.1%	86.2%	85.0%
	N	5**	5	5								
(b)	F	1.68± 0.18	2.86± 1.16	7.50± 6.80								
0.075% Asp	P	2.0±0	2.0±0	4.8±6.3								
	%	119.0%	69.9%	64.0%								
	N	3	3	3								
(c)	F	1.87± 0.42	2.60± 0.92	2.47± 1.03								
0.10% Asp	P	2.0±0	2.7± 1.2	2.0±0								
	%	107.0%	103.8%	81.0%								
	N	4	4	4	4	4	4	4	4	4	4	3
(d)	F	1.05± 0.19	2.65± 1.54	7.95± 4.48	5.98± 1.86	5.25± 3.19	3.85± 0.33	3.08± 1.08	4.20± 1.11	3.08± 1.10	3.45± 2.10	2.90±1.53
0.125% Asp	P	2.0±0	2.0±0	8.8±5.8	4.2±2.6	4.2±2.6	3.8±2.1	2.0±0	3.5±1.7	2.6±1.2	3.5±1.7	3.0±1.4
	%	190.5%	75.5%	110.7%	70.2%	80.0%	98.7%	64.9%	83.3%	84.4%	101.4%	103.4%
	N	5	5	5								
(e)	F	1.58± 0.18	2.34± 1.09	3.52± 1.68								
0.150% Asp	P	2.0±0	3.2±1.6	2.0±0								
	%	126.6%	136.8%	56.8%								

Continued...

Table 33 Series C, Experiment 5
(Con't)

GROUP		PREDIET	NUMBER OF DAYS ON TEST DIET								
SWEETENER	*	0	2-5	6-8	9	13	16	19	21	23	26	30-33
	N	2	3	3								
(f)	F	1.65± 0.25	4.10± 1.99	3.77± 0.86								
0.175% Asp	P	2.0±0	4.2± 0.3	2.0±0								
	%	121.2%	102.4%	53.1%								
	N	3										
(g)	F	1.27± 0.12										
Purina	P	2.0±0										
	%											

* N = number of samples
F = fluorometric determination (mg/100 ml)
P = paper chromatographic determination (mg/100 ml); values of < 4 taken as 2 mg/100 ml for calculation.
% = (P/F) 100
** one sample not analyzed by chromatography

animals and these data are summarized in Table 34. In the presence of synthetic cofactor the activity in tissue obtained from animals which had been consuming the 3.5% Phe 1.0% pCPA diets ranged from 25 to 32% of the Purina control activity. When compared to Purina control by unpaired t-tests, the Phe hydroxylase activity of groups (b) and (f), of (b) and (f) together, and of (a), (b) and (f) together were all highly significantly less than control activity ($p < .0001$). These data confirm that hepatic Phe hydroxylase is significantly inhibited by feeding guinea pigs a 3.5% Phe 1.0% pCPA diet. In addition the degree of enzyme inhibition was similar whether the 3.5% Phe 1.0% pCPA diets were sweetened with 8% sucrose or with Asp.

In summary, this experiment demonstrated that 3.5% Phe 1.0% pCPA test diets sweetened with Asp were associated with hyperphe when fed to adult female guinea pigs. In addition, these data suggest that a 0.175% Asp supplement resulted in the highest daily food intake among the diets evaluated.

Table 34 Series C, Experiment 5. Mean (±S.D.) hepatic phenylalanine hydroxylase activity in the presence of synthetic cofactor dimethyltetrahydropteridine.

Group Diet	Number of Animals	ACTIVITY μMoles Tyr/hr/g wet weight	ACTIVITY % Purina Control
(g) Purina Control	6	38.16±7.94	100%
(a) 3.5% Phe 1.0% pCPA 8% sucrose	2	12.09±3.86	31.7%
(b) 3.5% Phe 1.0% pCPA 0.075% Aspartame	5	10.86±2.80	28.4%
(f) 3.5% Phe 1.0% pCPA 0.175% Aspartame	3	9.42±1.68	24.7%

Series D, Experiment 1

Introduction

Guinea pigs require exogenous vitamin C in order to normally metabolize Tyr loads (Zannoni and LaDue 1960; LaDue and Zannoni 1961). Preliminary experiments in this laboratory indicated that guinea pigs fed diets supplemented with Phe and pCPA frequently had elevated plasma Tyr levels. Since ascorbic acid is rapidly converted to biologically inactive compounds in the presence of air and water (Sebrell and Harris 1967; Bender 1971; Lewin 1976), it seemed possible that during laboratory preparation of test diets a significant amount of the vitamin C may have been destroyed. These test diets therefore may not have provided sufficient vitamin C to permit the animals to metabolize an abnormally high Tyr load. The equivalent of a Tyr load was present in the guinea pigs fed Phe plus pCPA diets because Phe hydroxylase, which was inhibited to approximately 25% of normal activity, undoubtably was still able to convert considerable Phe to Tyr, therefore leading to Tyr accumulation and hypertyrosinemia.

In order to test this hypothesis the ascorbic acid content of Purina chow and laboratory prepared test diets was determined, and in addition, the effect of ascorbic acid supplements on the plasma PA and Tyr levels of animals

fed Phe and pCPA test diets was studied. The daily ascorbic acid dosage used was well above the minimum daily requirement of guinea pigs (Reid and Bieri 1972) and in the range found to be maximally effective in experimentally stressed guinea pigs (Veen-Baigent et al 1975; Yew 1973).

Methods

Samples of Purina chow and laboratory prepared test diets were homogenized (10% w/v) with cold 5% TCA and assayed in quadruplicate for ascorbic acid content by the α, α'-dipyridyl method.

Five (5) groups of Hartley guinea pigs were used in this experiment. Plasma was collected by the ocular method and Phe, PA, and Tyr assayed fluorometrically; plasma was also chromatographed on paper when sufficient volume was available. Daily food intake and body weight was recorded during the test diet periods.

The experiment was done in two parts, the first of which included two groups of guinea pigs.

Group (a-) (2.5% Phe 1.0% pCPA diet, no vitamin C). Seven (7) animals were obtained from Carworth, bled for baseline Phe and Tyr while consuming standard chow, and given a diet supplemented with 2.5% Phe 1.0% pCPA 4% sucrose and 4% lettuce when 40 days of age. After 16 days on this diet the lettuce was replaced with 5% cabbage and the sucrose supplement increased to 5% to try to improve the test diet

palatability. Plasma was collected every two to four days. During the first 15 days on the test diet no vitamin C was given, except that provided by the test diet. During this period the animals were bled on a number of days. On the fifteenth day, after being bled, four of the animals were injected with ascorbic acid (see group (a+) below) and the remaining three animals were injected with equivalent volumes of water (day 15, 1 ml/300 g; day 16 and 17, 2 ml/300 g). After blood was collected on day 17, these three animals were also given ascorbic acid and therefore also entered group (a+) (see below).

Group (a+) (2.5% Phe 1.0% pCPA diet, plus vitamin C). When the animals in group (a-) were given vitamin C supplements they formed group (a+). In this group all animals received daily IP ascorbic acid injections of 20 mg/300 g (1 ml/300 g) on day 15 and 40 mg/300 g (2 ml/300 g) from day 16 on. This group was also included in Series C, Experiment 2 as group (a), and is described in detail there. Briefly, the animals continued on the 2.5% Phe 1.0% pCPA diet used in group (a-) but were given daily injections of freshly prepared neutralized ascorbic acid. All seven animals remained on this regimen for 22 days, making a total period of 36 days on the test diet. Blood was collected for PA, Phe and Tyr estimation on a number of days. This design allowed comparison of the same animals consuming the 2.5% Phe 1.0% pCPA test diet, first

without ascorbic acid and subsequently with ascorbic acid supplementation.

The second part of this experiment compared the effects of ascorbic acid supplementation on groups of animals eating 3.5% Phe 1.0% pCPA test diets.

Group (b+) (3.5% Phe 1.0% pCPA diet, plus vitamin C). The five animals in Series C, Experiment 1, group (c) made up this group and they are described in detail above. Briefly, these guinea pigs were fed a diet supplemented with 3.5% Phe 1.0% pCPA 7% sucrose and 3% cabbage for 25 days. During the experiment the animals received daily oral administration of 50mg/300g of freshly prepared ascorbic acid in a sucrose solution (4 g/50 ml). Plasma was collected at various intervals during the test diet period.

Group (b-1) (3.5% Phe 1.0% pCPA diet, no vitamin C). The 4 guinea pigs used in this group were run simultaneously with group (b+), were purchased at the same time from the same supplier (Sunrise Mousery), and were the same age when given the test diet (112± 3 days) as the animals in group (b+). They were treated identically to the animals in group (b+) except they did not receive ascorbic acid. From the third day of the experiment the animals received orally an equivalent volume (1 ml/300 g) of the sucrose vehicle solution (4 g/50 ml). Animals in both groups (b+) and (b-1) remained on the 3.5% Phe 1.0% pCPA 7% sucrose 3% cabbage test diet for 25 consecutive days. Both groups (b+) and (b-1) were bled on days 8, 11, 17, 23, and 25 of the experiment.

Group (b-2) (3.5% Phe 1.0% pCPA diet, no vitamin C). All six animals in this group had been included in Series C, Experiment 1 or in group (b-1) above; following these experiments, which ran concurrently, the animals consumed Purina chow for over one month prior to forming group (b-2). The animals used previously in Series C, Experiment 1 included one from each of groups (a), (d) and (e); three were included in group (b-1). All animals in group (b-2) were obtained from Sunrise mousery and except for one (124± 2 days), were 162 ± 3 days old when the experiment began. The animals were given a diet supplemented with 3.5% Phe 1.0% pCPA 7% sucrose and 3% cabbage for 21 days. Each animal received daily oral administration of 1 ml/300 g of sucrose solution (4 g/50 ml). Animals were bled 7, 17, and 21 days after the test diet was presented.

Results

Ascorbic acid content of diets. The manufacturer claims that Purina chow contains at least 1 mg of ascorbic acid per g of diet; measurements in this laboratory detected a mean of 1.32 mg/g in Purina chow. In contrast to Purina chow, test diets supplemented with pCPA and/or Phe contained only about 25% of this amount of ascorbic acid. The 3.5% Phe 1.0% pCPA diet contained 0.336 mg/g, while diets supplemented with 3.5% Phe but no pCPA had a mean ascorbic

acid content of 0.318% mg/g. These data demonstrate that approximately 75% of the ascorbic acid in Purina guinea pig chow is lost during the laboratory preparation of test diets.

Plasma phenylalanines and tyrosine. The animals in group (a-) initially consumed a diet supplemented with 2.5% Phe and 1.0% pCPA without additional ascorbic acid administration. After 15 or 17 days on this test diet, the animals were given daily ascorbic acid injections forming group (a+). The effect of the vitamin C supplement on plasma PA and Tyr levels in these animals is illustrated in Figure 14. In the absence of vitamin C administration the animals had substantial and persistant hypertyrosinemia after 4 days on the test diet. Administration of 40 mg/300 g/day of vitamin C however, dramatically reduced the magnitude and the variation in plasma Tyr levels. In order to test the significance of the change in Tyr levels following vitamin C administration, paired t-tests were used to compare the mean Tyr level of each animal prior to and during the period of ascorbic acid treatment. The mean Tyr level of all animals prior to ascorbic acid treatment was 8.37 mg/100 ml and the level during the period of vitamin administration was 2.50 mg/100 ml; this difference is highly significant ($t = 6.020$, $df = 6$, $p < .001$, two-tailed). The ability of ascorbic acid to reduce plasma Tyr levels was apparent after only 2 days of supplement; the four animals given 20 mg/300 g of vitamin C on day 15 and 40 mg/300 g on day 16 had

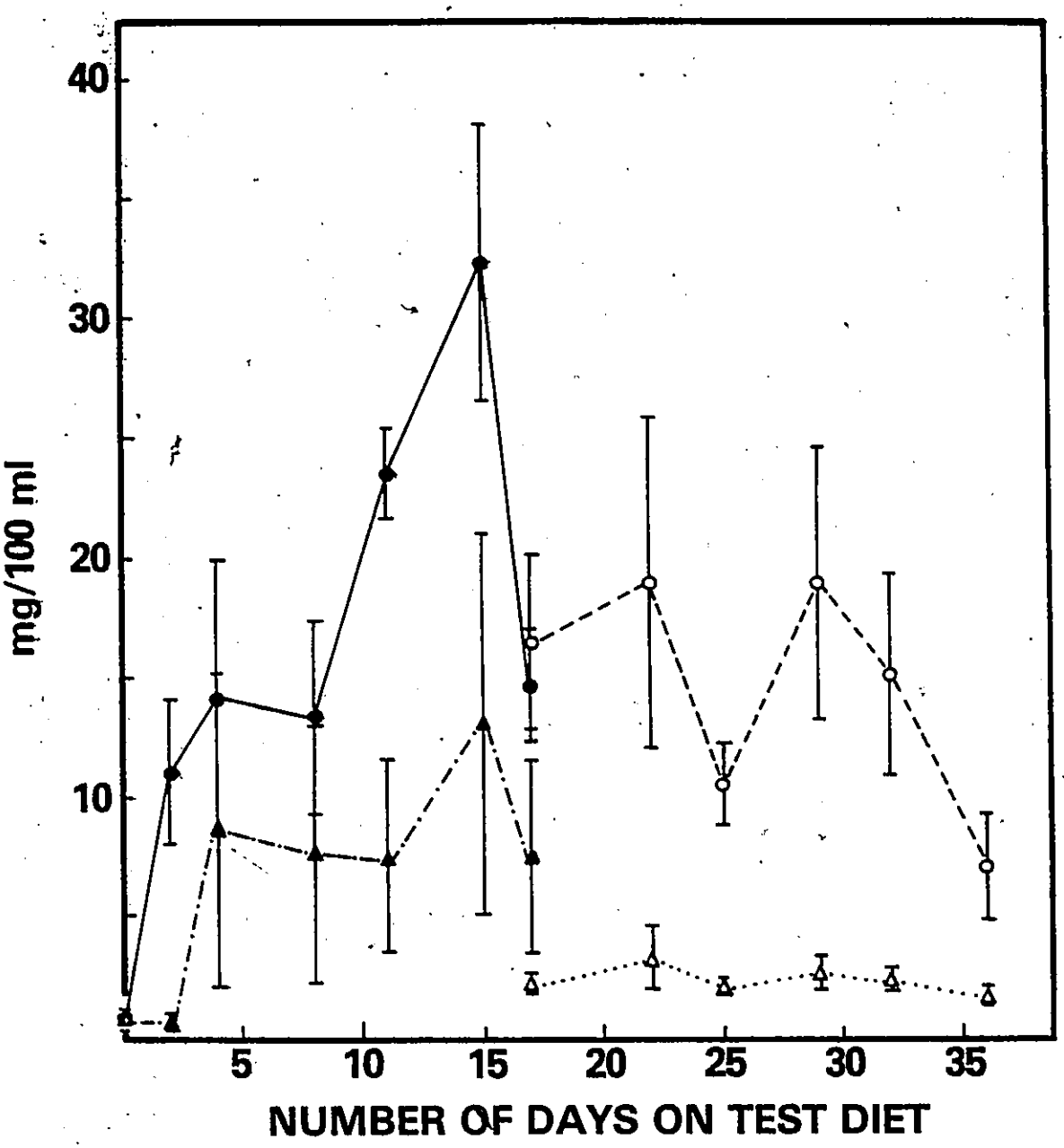


Figure 14. Series D, Experiment 1. Mean (±S.D.) plasma phenylalanines (circles) and tyrosine (triangles) without ascorbic acid and during ascorbic acid supplementation (open circles and triangles).

significantly lower Tyr levels on day 17 than those animals not given the vitamin (unpaired $t = 2.753$, $df = 5$ $p < .05$, two-tailed) (Figure 14).

Although only two samples from animals not receiving the vitamin C treatment were available for paper chromatography, one of them had a Tyr level of 27 mg/100 ml. The single sample chromatographed from an animal in group (a+) receiving vitamin C had an undetectable amount of Tyr (ie less than 4 mg/100 ml).

Compared to prediet Phe levels, the test diet clearly elevated plasma PA levels (Figure 14). Plasma PA levels however, did not appear to be affected as dramatically by vitamin C treatment as were the Tyr levels (Figure 14), although a paired t-test of the means of each animal from before and during vitamin C treatment was significant (mean before = 19.44 mg/100 ml, mean during = 14.36 mg/100 ml, $t = 3.849$, $df = 6$, $p < .01$, two-tailed). The paper chromatographic Phe values were 6 and 10 mg/100 ml before, and 16 mg/100 ml during vitamin C treatment.

The second part of this experiment included three groups of animals fed test diets supplemented with 3.5% Phe and 1.0% pCPA. One group (b+) received oral supplements of 50 mg/300 g/day of vitamin C, while the remaining groups (b-1), (b-2) received only the vehicle without vitamin C. Table 35 summarizes the plasma PA and Tyr levels (fluorometric) in these three groups before and during the test diet period.

All three groups had clear elevations of plasma PA during the experimental period when compared to prediet Phe levels. The plasma PA data from the test diet period was pooled across the two non-vitamin groups (b-1, b-2) using the mean for each animal for each time interval, transformed to logarithms, and subjected to ANOVA with repeated measures. This analysis did not reveal significant effect for vitamin C treatment ($F = 0.165$, $df = 1, 10$) or for the number of days on the test diet ($F = 0.647$, $df = 3, 26$). Plasma Phe levels, as estimated by paper chromatography, were lower in the nonsupplemented group (b-1), mean of 18 samples, 9.7 mg/100 ml than in the supplemented animals (b+), mean of 11 samples, 15.6 mg/100 ml.

Plasma Tyr levels for groups (b+), (b-1) and (b-2) are also shown in Table 35. Inspection of these data suggest that during the test diet period animals not treated with vitamin C had higher and more variable Tyr levels than the group given vitamin C (b+). The plasma Tyr data from the test diet period were handled in the same manner as the PA data; the Tyr data were pooled across groups (b-1) and (b-2) and analysed by ANOVA with repeated measures. Analysis of the logarithm transformed data indicated insignificant effects for the number of days of test diet consumption ($F = 0.289$, $df = 3, 27$) and vitamin C treatment ($F = 1.198$, $df = 1, 10$). It was found however, that the variances in Tyr levels between vitamin

Table 35 Series D, Experiment 1. Mean (±S.D.) total phenylalanines and tyrosine as a function of the number of days on test diet: effect of ascorbic acid administration. Groups (b+), (b-1), and (b-2), fluorometric determinations, mg/100 ml.

	DIET GROUP (3.5% Phe 1.0% pCPA)	NUMBER OF DAYS ON DIET						
		0 Prediet	7 or 8	11 or 14	17	21 or 23	25	Total Test Diet
Plasma Total Phenylalanines (mg/100 ml)	(b+) 50 mg/300g Ascorbic Acid	(4)* 1.28 ±0.21	(4) 23.42 ±7.31	(5) 18.32 ±7.16	(5) 28.98 ±9.20	(5) 28.96 ±6.92	(2) 26.35 ±2.90	(21) 25.13 ±8.06
	(b-1) no Ascorbic Acid	(4) 1.60 ±0.23	(4) 20.92 ±0.89	(4) 14.65 ±2.63	(4) 20.72 ±5.67	(4) 28.52 ±7.83	(4) 24.42 ±2.68	(20) 21.85 ±6.26
	(b-2) no Ascorbic Acid	(5) 1.46 ±0.26	(3) 36.53 ±12.74	(5) 40.58 ±9.78	(3) 34.43 ±9.36	(6) 28.75 ±14.26	(0)	(17) 34.61 ±11.98
Plasma Tyrosine (mg/100 ml)	(b+) 50 mg/300 Ascorbic Acid	(4) 0.98 ±0.24	(4) 5.22 ±0.79	(5) 3.92 ±0.52	(5) 5.20 ±1.43	(5) 5.24 ±2.13	(2) 5.75 ±2.33	(21) 4.96 ±1.45
	(b-1) no Ascorbic Acid	(4) 1.40 ±0.28	(4) 4.18 ±1.01	(4) 11.85 ±10.92	(4) 3.30 ±1.02	(4) 13.68 ±17.84	(4) 4.65 ±0.51	(20) 7.53 ±9.44
	(b-2) no Ascorbic Acid	(5) 1.24 ±0.33	(3) 11.20 ±11.95	(5) 7.52 ±6.49	(4) 11.18 ±9.73	(6) 7.73 ±5.40	(0)	(18) 9.02 ±7.43

* Number of plasma samples.

C treated and untreated groups over the whole test diet period differed significantly. The variance for group (b+) was 3.69 and for (b-1, b-2) it was 97.81; comparison of these variances using an F-test revealed a highly significant difference ($F_{6,4} = 26.500$ $p << .001$). This difference in variances, reflected in the high standard deviations of Tyr in the vitamin C untreated groups (Table 35), could have obscured the significance in the ANOVA of an effect of vitamin C treatment on plasma Tyr.

Paper chromatography of plasma samples from groups (b+) and (b-1) revealed a higher mean Tyr in the untreated group: 6.9 mg/100 ml compared to 5.4 mg/100 ml. Three of the samples chromatographed from (b-1) had Tyr levels of 12, 25, and 27 mg/100 ml. In addition, one sample obtained from the severed neck as part of another study of an animal in group (b-2) had a Tyr level of 27 mg/100 ml. Except for one sample from an animal in the vitamin C group (paper Tyr = 12 mg.100 ml), all Tyr levels from (b+) animals were less than 8 mg/100 ml.

In summary, the first part of the experiment showed that both PA and Tyr were significantly lower when the animals received supplemental ascorbic acid. The second part of the study also suggested Tyr was lower in the vitamin C-treated animals, although the significantly higher variability of plasma Tyr in these animals may have made the difference in Tyr levels impossible to detect by ANOVA.

Body weight and food intake. When initially given the test diets all groups, except (a+) which had already been eating the test diet for 15 days, displayed the decrease in food intake characteristic of guinea pigs presented with Phe and pCPA supplement diets. By the end of the study period all groups were eating the test diets at comparable rates. The mean intakes in g/24 hours for the final day of the study for each group were as follows: (a-) 26.4, (a+) 27.6, (b+) 25.8, (b-1) 22.5, (b-2) 25.0. In contrast to previous experiments, animals in groups not supplemented with vitamin C failed to gain weight during the test diet period. Groups (a-), (b-1) and (b-2) lost 3.1%, 4.2% and 13.9% of the prediet weight during the experiment. The groups receiving vitamin C however, each gained weight during the experiment, 16.5% and 4.1% of their prediet weights in groups (a+) and (b+), respectively.

Series D, Experiment 2

Introduction

The data presented in Series D, Experiment 1 demonstrated that guinea pigs maintained on laboratory-prepared test diets required daily ascorbic acid supplements in order to minimize the magnitude and variability of hypertyrosinemia. The vitamin C supplement of 50 mg/300 g was in excess of the published minimum daily requirement for the guinea pig, even under conditions of surgical stress (Reid and Bieri 1972; Veen-Baigent et al 1975; Yew 1973). No signs of scurvy were observed in the animals not provided with supplemental ascorbic acid, although these animals did lose weight. Further studies were undertaken to determine whether the daily oral ascorbic acid supplements affected tissue ascorbic acid levels of animals fed Phe and pCPA test diets. Hepatic ascorbic acid content was compared among three groups of guinea pigs: Purina chow fed controls and animals consuming a 3.5% Phe 1.0% pCPA test diet which received either daily ascorbic acid supplements or administration of the sucrose vehicle but without ascorbic acid.

Methods

Animals were placed on the appropriate diets, with

or without supplemental ascorbic acid, bled regularly for PA, Phe and Tyr determinations (fluorometric and paper chromatographic) and killed by decapitation. Livers were then quickly removed, weighed, and frozen on solid CO_2. Four or 5 days after death samples of frozen liver tissue were homogenized 5% (W/V) in ice cold 5% TCA and assayed in duplicate for ascorbic acid content by the α,α-dipyridyl method.

The following 3 groups of guinea pigs were included in this experiment:

Group (a): Purina chow control. 12 animals were included; 7 were obtained from M. & L. Farms, 4 from Canadian Breeding Laboratories and one from Carworth. At the time of death the mean age was 151.5 days (range 121-317 days). Prior to entering group (a) of this experiment, 9 of the 12 guinea pigs participated in previous studies as follows: six animals comprised the Purina-fed control group of Series C, Experiment 5, and two served as the Purina controls in Series C, Experiment 1. An additional animal was included in the following experiments: Series C, Experiment 2, groups (b) and (i), and Series D, Experiment 1, groups (a-) and (a+); this animal then consumed Purina chow for 114 consecutive days prior to being killed as part of the current study. The remaining 3 animals ate only Purina chow while in the laboratory and were not included in any other studies. Purina chow provided the only source of

vitamin C for the animals in group (a).

Group - (b): 3.5% Phe 1.0% pCPA test diet + 50 mg/ 300 g/ day ascorbic acid. A total of 22 animals were included in this group, all of which were also included in previous experiments as indicated in Table 36. Details concerning previous handling of these animals are provided under the relevant experiments already described (Table 36). All animals had consumed laboratory-prepared diets (with or without added Phe and pCPA) and received daily ascorbic acid supplements for a minimum of 22 consecutive days at the time of death; the final diet on all cases was supplemented with 3.5% Phe 1.0% pCPA and additional flavourings, as shown in Table 36. All animals received freshly prepared oral ascorbic acid in a total dose of 50 mg/300g/day. Nine (9) of the animals were given ascorbic acid, 25 mg/300 g/dose, twice per day and the remaining 13 received the 50 mg/300 g as a single daily administration (Table 36). Ascorbic acid was delivered in a sucrose solution (4 g/50 ml) at a volume of 1 ml/300 g or 0.5 ml/300 g when two doses per day were given.

On the final day of the experiment the animals received one half the total daily dose of vitamin C in the morning and were then killed during the afternoon.

Group (c): 3.5% Phe 1.0% pCPA diet, no ascorbic acid supplement. Seven guinea pigs were used in this group. Six (6) of them were included in Series D, Experiment 1 as

Table 36 Series D, Experiment 2, Group (b). Summary of animals included in group (b). 3.5% Phe 1.0% pCPA test diet plus 50 mg/300 g/day ascorbic acid supplement.

PREVIOUS EXPERIMENT			Number of Animals	Diet When Killed	# days on diet at death	Age at death (days)	Ascorbic Acid: # doses/day
Series	Experiment Number	Group					
C	1	(c)	3	3.5% Phe 1.0% pCPA 7% sucrose 3% cabbage	25	137	1x
C	2	(e)	4	3.5% Phe 1.0% pCPA 8% sucrose	29	88	2x
C	4	-	3	3.5% Phe 1.0% pCPA 8% sucrose 2% cabbage	31	136	2x
C	4	-	2	3.5% Phe 1.0% pCPA 8% sucrose	31	136	2x
C	5	(a)	2	3.5% Phe 1.0% pCPA 8% sucrose	33	123	1x
C	5	(b)	5	3.5% Phe 1.0% pCPA 0.075% Aspartame	6	93	1x
C	5	(f)	3	3.5% Phe 1.0% pCPA 0.175% Aspartame	6	93	1x

group (b-2) and were killed at end of that study. The additional animal was the same age and was treated identically to the 5 older pigs in Series D, Experiment 1 group (b-2) but was not included in the presentation of that experiment only because no plasma samples were available for amino acid analysis. At the conclusion of that experiment, after 24 days on the test diet (3.5% Phe 1.0% pCPA 7% sucrose 3% cabbage), all animals were killed and hepatic ascorbic acid content determined 5 days after death. During the course of the experiment each animal received a single daily oral administration of 1 ml/300 g of sucrose solution, 4 g/50 ml. When killed, 6 animals were 200 days old and one was 163 days of age.

Daily food intake and body weight were monitored in groups (b) and (c), as described in the relevant previous experiments.

Results

Hepatic Ascorbic Acid. In groups (a) and (b) hepatic ascorbic acid levels were determined on frozen tissue samples 4 or 5 days after the animals were killed. Comparison of liver ascorbic acid content within each of these groups showed that no significant difference existed between samples assayed 4 days or 5 days after death [Purina (a), 4 days mean 53.24 mg/100 g wet weight; 5 days mean 54.10 mg/100 g wet weight; unpaired $t = 0.06184$, $df = 10$, and (b)

3.5% Phe 1.0% pCPA + Vitamin C, 4 days mean 21.69 mg/100 g wet weight, 5 days mean 21.91 mg/100 g wet weight, unpaired t = 0.05125, df = 20]. Since the day of assay did not significantly influence the levels of measured ascorbic acid, the data from both days, within group (a) and within group (b), were each pooled for further analyses.

Figure 15 illustrates the group mean and individual levels of hepatic ascorbic acid found in groups (a), (b) and (c). Daily administration of 50 mg/300 g of ascorbic acid, group (b), was associated with a significantly higher hepatic ascorbic acid content (21.77 ± 9.44 gm/100 g wet weight) when compared to that of animals also consuming the 3.5% Phe 1.0% pCPA diet, but without supplementary vitamin C, [group (c), 7.08 ± 2.82 mg/100 g wet weight; unpaired t = 4.0178, df = 27, p << .005, two-tailed]. In addition, Purina fed animals had a hepatic ascorbic acid level (53.67 ± 22.97 mg/100 g wet weight) significantly greater than the animals fed 3.5% Phe 1.0% pCPA diet and given daily vitamin C (21.77 ± 9.44 mg/100 g wet weight, t = 5.7400, df = 32, p << .005 two-tailed).

Within group (b), it was found that administration of the 50 mg/300 g/day of ascorbic acid as a single daily dose was associated with a significantly higher hepatic ascorbic acid content (25.94 ± 10.09 mg/100 g wet weight) than when the same total daily dose of ascorbic acid was given as two 25 mg/300 g doses (15.75 ± 3.44 mg/100 g wet

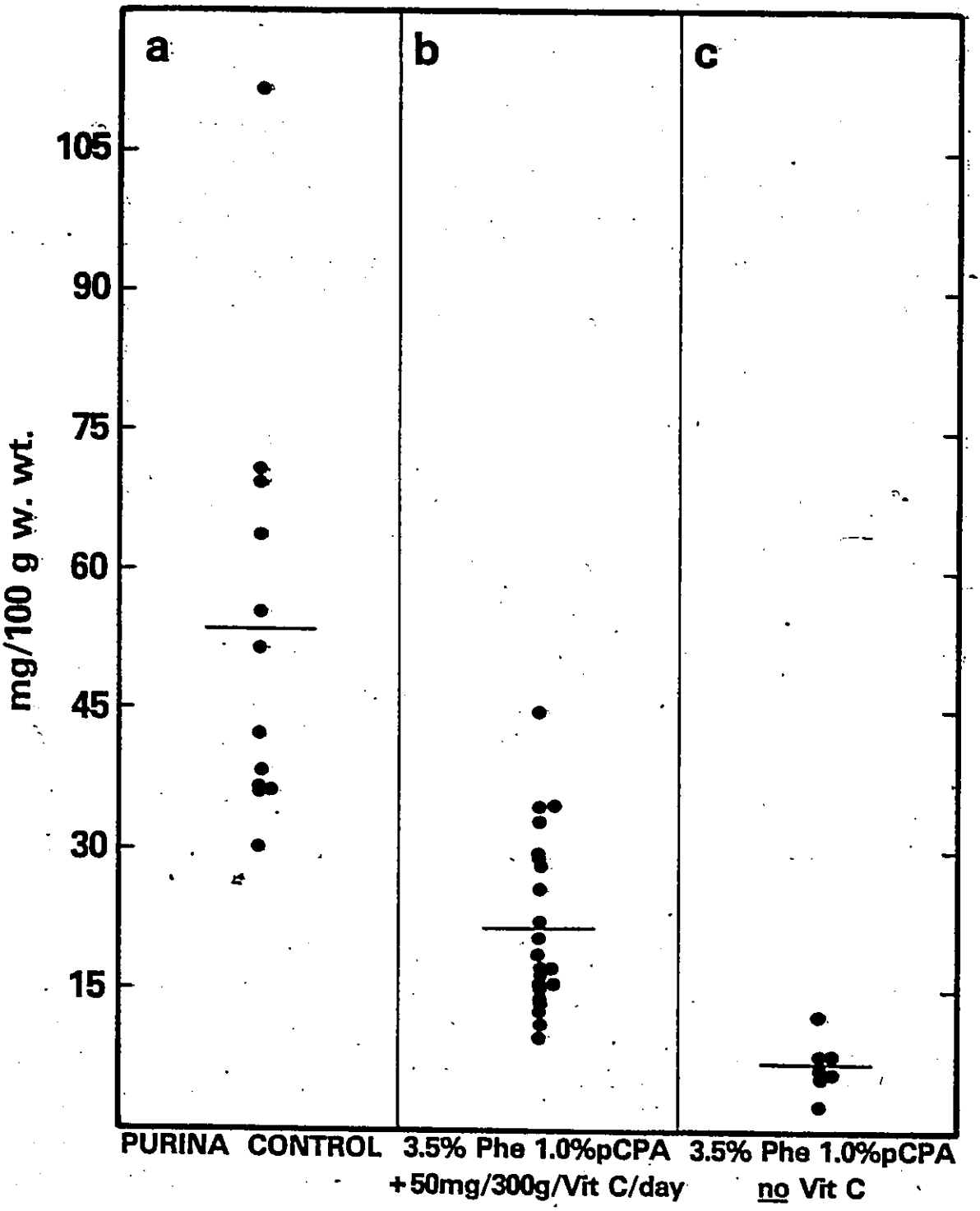


Figure 15. Series D, Experiment 2. Hepatic ascorbic acid content (mg/100 g wet weight) for each subject. Horizontal bar indicates mean for each group.

weight, t = 2.8981, df = 20, $p < .01$, two-tailed). The hepatic ascorbic acid content of the animals which received the single daily dose of vitamin C however, was still significantly less than the Purina-fed control levels (t = 3.9628, df = 23, $p < .01$, two-tailed). Within groups (a) and (b), neither age, nor presence or absence of cabbage or Aspartame in the diets, significantly affected hepatic ascorbic acid levels.

Plasma PA, Phe, and Tyr. Plasma was collected and analyzed throughout the course of the experiments in groups (b) and (c). Results from samples taken within 2 days of death are shown in Figure 16; earlier blood samples had PA, Phe, and Tyr levels in the range found in other studies with the 3.5% Phe 1.0% pCPA diet and are included in previous experiments (see Series D, Experiment 1 and Table 36 for reference). Purina fed animals had normal plasma Phe and Tyr levels and all of these samples had undetectable Phe and Tyr levels by paper chromatography. Both groups fed 3.5% Phe 1.0% pCPA test diets had markedly elevated PA levels during the final 2 days of the study. Plasma Phe, as measured by paper chromatography was elevated in group (b); it was not possible to measure Phe or Tyr chromatographically in group (c) because sufficient plasma was not available. Plasma PA levels during the final two days of the study were marginally greater among the animals not given vitamin C compared to those given the vitamin (28.8 mg% vs 19.3 mg%, t = 2.0908,

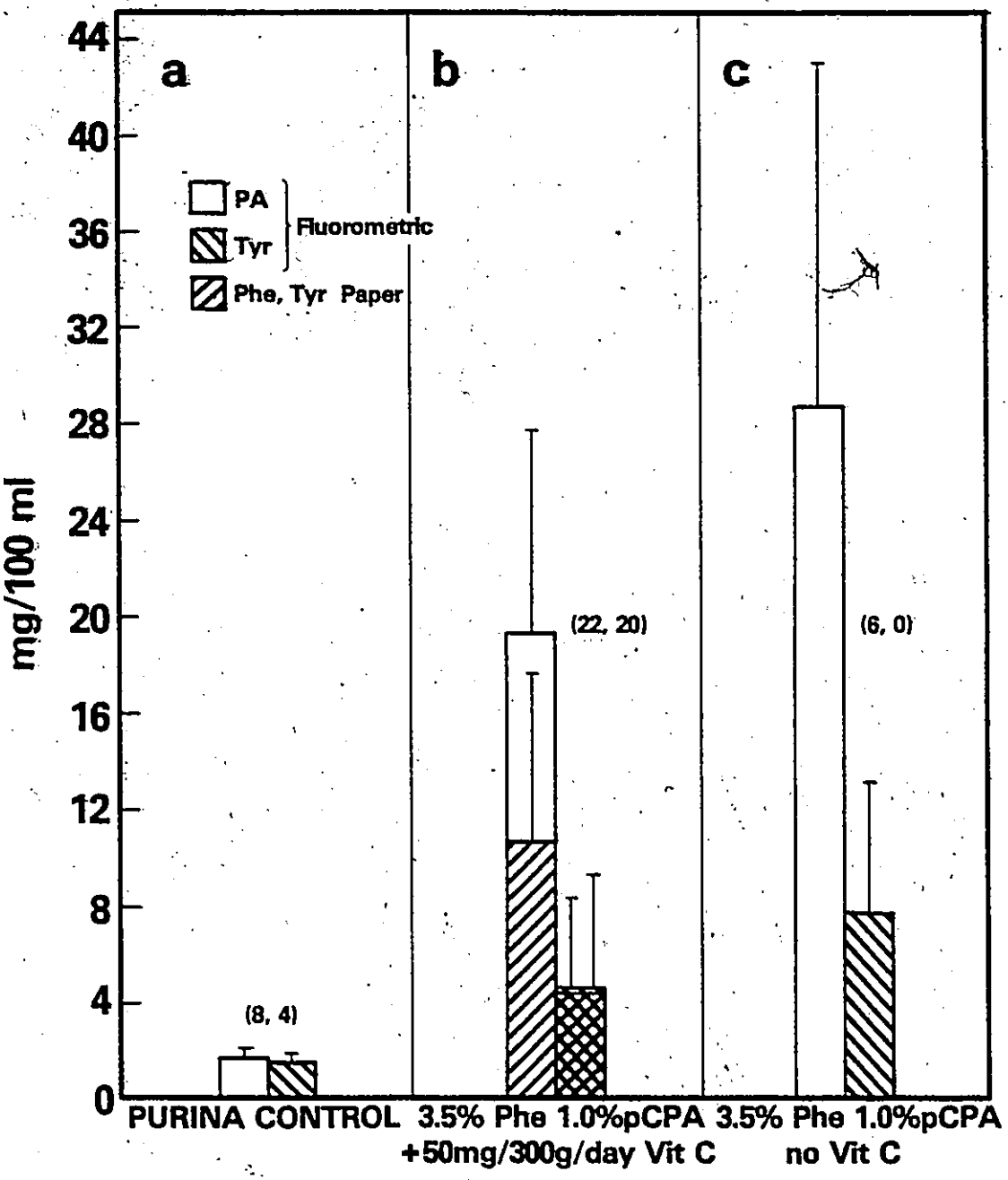


Figure 16. Series D, Experiment 2. Mean (+S.D.) plasma phenylalanines and tyrosine in each group. Numbers in parentheses indicate number of samples analyzed by fluorometry and by paper chromatography, respectively.

df = 26, $p < .05$, two-tailed). Tyr, as determined fluorometrically, did not differ significantly during this interval between these groups, although the trend was toward higher levels in the unsupplemented group (7.73 mg% vs 4.56 mg%, $t = 1.6634$, df = 26).

Body and liver weight. Body weights during the course of the study are included in previous experiments (see Series D, Experiment 1 and Table 36 for reference). Both groups lost weight when initially presented with the test diets. Only group (c) however, continued to lose weight throughout the test diet period unlike the ascorbic acid-treated animals which had a net gain of weight. Body weight and liver weights on the final day of the study are illustrated in Figure 17. The Purina control animals were both significantly heavier and had significantly greater liver weights than either of the test diet groups ($p << .005$, two-tailed), which in turn did not differ significantly from one another. The animals in group (c) were both older and heavier when the study began, however the animals receiving ascorbic acid supplements exceeded their weight when the experiment ended.

Food intake. Data on food intake were collected for the animals in groups (b) and (c); these data are included in previously described experiments (see Table 36 and Series D, Experiment 1). Both groups had comparable levels of food consumption.

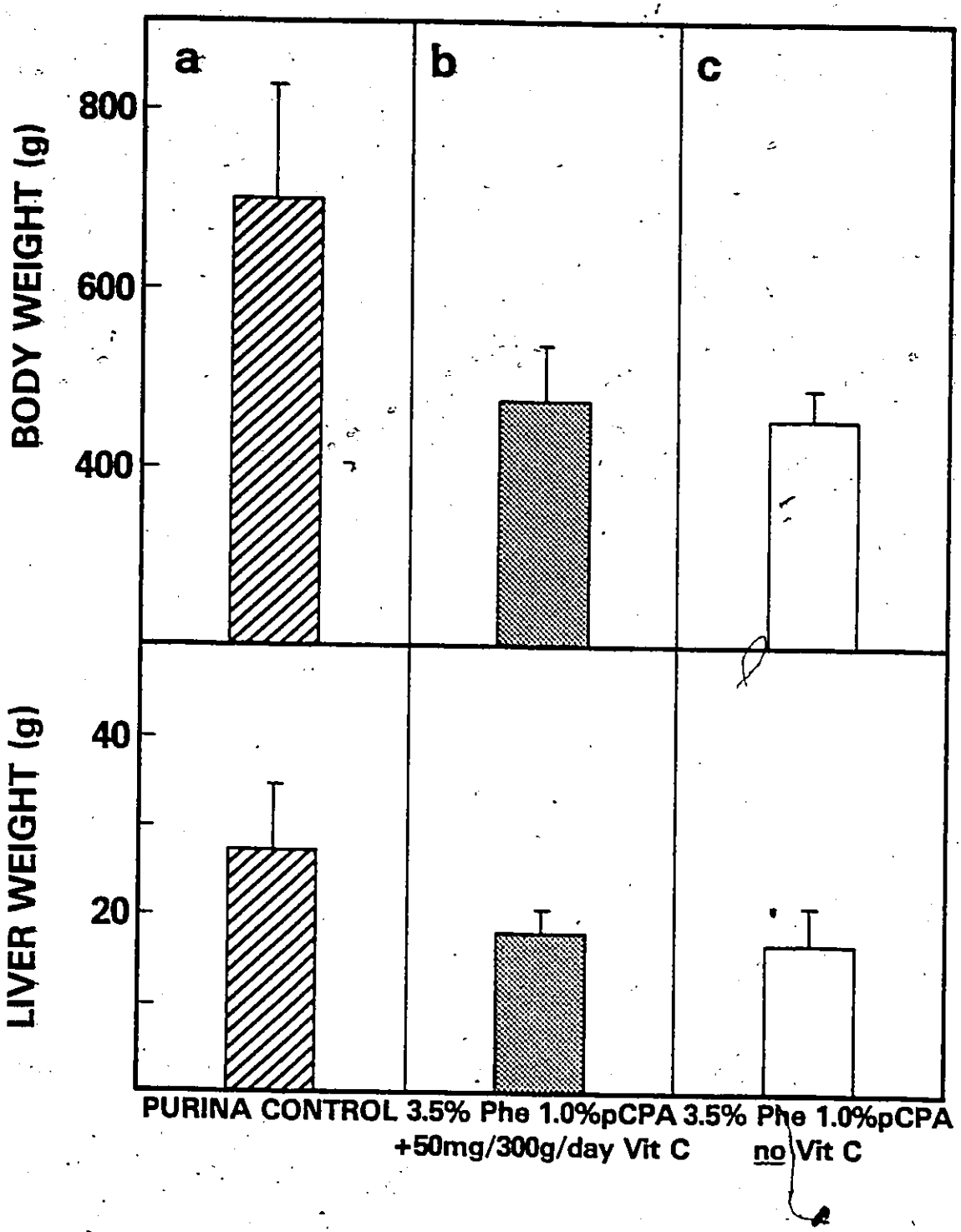


Figure 17. Series D, Experiment 2. Mean (+S.D.) body weight and liver weight of each group.

Series E, Experiment 1

Introduction

The normal concentrations of Phe and Tyr in human fetal plasma near term are between 1.5 and 2 times higher than corresponding maternal Phe and Tyr levels (Ghadimi and Pecora 1964; Matsuda et al 1977). Although the absolute levels of Phe are greatly increased in both fetal and maternal plasma in cases of maternal PKU, the fetal:maternal ratio of plasma Phe is not appreciably different from that found in normal pregnancies (Bovier-Lapierre et al 1974; Bush and Dukes 1975; Cockburn et al 1972; Huntley and Stevenson 1969; Thomas et al 1971). Christensen and Streicher (1948) reported that in the near term guinea pig the fetal:maternal ratio of amino acids (α-amino nitrogen) was about 5:1. In order to produce fetal blood Phe levels similar to those found clinically in maternal PKU, maternal Phe levels in an experimental model would be considerably lower than those observed clinically if the fetal:maternal ratio were in fact 5:1. That is, to reproduce the fetal plasma levels expected in a woman with a Phe level of 15 mg/100 ml, ie about 21 to 30 mg/100 ml in fetal blood, maternal Phe levels would need to be elevated to only about 6 mg/100 ml in an animal with a 5:1 fetal to maternal ratio. The purpose of

the following experiment was to accurately determine the fetal:maternal ratios of Phe and Tyr at different stages of pregnancy in normal guinea pigs. This information is obviously of value in defining the appropriate maternal Phe level in a model of maternal PKU utilizing the guinea pig.

Materials and Methods

The 11 females used in this study were Hartley strain guinea pigs, 10 of which were obtained from Carworth and one from M. & L. Farms. All animals were mated with Hartley males. Females ranged from 61 to 104 days of age on the first day of pregnancy.

The mating procedure is described above and the day sperm were detected was designated as day 1 of gestation. In one case the mating was missed and the female delivered on day 66 (by calculation).

With one exception, all animals were fed only Purina chow. One animal was given a 3.5% Phe 1.0% pCPA 8% sucrose diet on gestation day 2, but was returned to Purina chow on day 12 because of poor food intake; this pig was then killed at 65 days gestation.

Pregnant animals were killed either by an overdose of ether (7) or by decapitation (4). Maternal blood was then collected by heart puncture (8 animals), from the severed neck (2 animals) or from a leg vein (1 animal). The abdomen was quickly opened and the fetuses weighed, examined

and blood collected from either the umbilical vessels or by cardiac puncture.

Maternal and fetal plasma samples were assayed in duplicate for Phe and Tyr by the fluorometric methods.

Animals were killed at various times between gestation day 38 and day 67, as indicated in Table 37.

Results

All pregnant females remained healthy and gained weight steadily throughout pregnancy. All fetuses were alive and morphologically normal. One resorption was detected, in a female killed on day 45 of gestation.

Table 37 summarizes the findings. Fetal-maternal ratios of Phe were less than 2:1 from 38 to 46 days gestation and rose to between 1.9 and 2.7 by the final week of gestation. Fetal plasma Phe was always found to be greater than the maternal level and the mean ratio over the entire period under study was 1.76 (Table 37). Within 12 hours of birth, newborn Phe levels had decreased to slightly less than maternal levels (Table 37).

Fetal-maternal ratios of Tyr were generally lower than those of Phe, although absolute values of both maternal and fetal Tyr tended to be slightly higher than those of Phe. At 38-39 days, the fetal Tyr levels were less than the comparable maternal levels, rose to nearly equal maternal Tyr at 45-46 days, and then ranged from 1.2 to 1.5 times

Table 37 Series E, Experiment 1 Maternal and fetal plasma levels of phenylalanine and tyrosine, fluorometric measurement.

Gestational Age (Days)	NUMBER		PHENYLALANINE mg/100 ml (mean ±S.D.)			TYROSINE mg/100 ml (mean ±S.D.)		
	Pregnant Females	Fetuses	Maternal	Fetal	Fetal/Maternal Ratio	Maternal	Fetal	Fetal/Maternal Ratio
38-39	2	5	1.40 (1.5, 1.3)*	2.26 ±0.49	1.61	3.20 (2.0, 4.4)	2.24 ±0.85	0.70
45-46	2	5	2.40 (2.6, 2.2)	2.56 ±0.15	1.07	2.45 (3.0, 1.9)	2.66 ±0.22	1.09
61	3	9	0.90 ±0.10	2.44 ±0.26	2.71	1.37 ±1.67	2.06 ±1.88	1.50
65	1	2	0.8	2.20 (2.2, 2.2)	2.75	3.9	4.85 (5.1, 4.6)	1.24
67	2	6	1.05 (1.0, 1.1)	2.03 ±0.25	1.93	2.45 (2.6, 2.3)	3.52 ±0.37	1.44
Pregnancy Mean			1.32	2.32	1.76	2.42	2.74	1.13
66 newborn, less than 12 hours old	1	3	1.3	1.10 ±0.10	0.85	2.6	3.37 ±1.02	1.30

* Individual values are shown when only 2 samples were available.

maternal levels during the final week of pregnancy. The mean fetal:maternal ratio for Tyr throughout the study interval was 1.13. In contrast to Phe, the newborn animal's Tyr level remained greater than the maternal level.

Series E, Experiment 2

Introduction

Studies of untreated pregnant guinea pigs demonstrated that maternal hyperphe must be in the range of 15 mg/100 ml to reproduce the degree of hyperphe found in the human fetus at risk of in utero damage (Series E, Experiment 1). The primary objective of the work presented in Appendix A and Series B through D was to define the conditions necessary to induce stable hyperphe accompanied by only minimal elevation of Tyr in adult female guinea pigs. Having achieved this objective, it became possible to study the effects of hyperphe on the pregnant guinea pig. There are no other reports of the effects on early pregnancy of induced hyperphe without concomitant hypertyrosinemia. Initial studies in this laboratory therefore examined the effects of such hyperphe on early pregnancy in the guinea pig. It was found that pregnant guinea pigs fed Phe and pCPA supplemented diets aborted their pregnancies. There are a number of factors which may have contributed to this pregnancy wastage and in the following study the roles of diet composition, hyperphe, maternal food intake, and the vulnerability of embryonic implantation have been considered.

Methods

All animals used were Hartley strain guinea pigs from the

same original breeding stock; one male and one female were obtained from Carworth and four males and 24 females from M. & L. Farms. The animals were housed one per cage as described above and the pregnancies included in this study were the first for each guinea pig.

Timed pregnancies were obtained as previously described and pregnant females were isolated on the day pregnancy was detected. Pregnant females were given various test diets and ascorbic acid supplements as described below. At intervals before and during pregnancy heparinized plasma was collected from the saphenous vein and assayed in duplicate for Phe, PA, and Tyr by the fluorometric methods. Phe and Tyr were also estimated by paper chromatography when sufficient plasma was available. $FeCl_3$ tests for phenylketones were performed when fresh urine was available. Daily body weight and food intake were monitored. Pregnant animals were divided into the following six treatment groups which are summarized in Table 38.

Group (a): Six (6) guinea pigs ate Purina chow until gestation day 10 or 11 when they were given a 3.5% Phe 1.0% pCPA 8% sucrose supplemented test diet. Daily ascorbic acid treatment consisted of 50 mg/300 g body weight orally. Plasma was collected for determination of baseline Phe and Tyr levels while the animals consumed Purina chow and PA and Tyr levels were estimated on a number of occasions during the test diet period.

Table 38 Series E, Experiment 2 Summary of experimental groups†**

GROUP	ANIMALS		DIETS			Ascorbic acid during pregnancy
	Number	Age on gestation day 1*	Before Pregnancy	Test Diet (gestation day begun)	Change during pregnancy	
(a)	6	72-91	Purina chow	3.5% Phe 1.0% pCPA 8% suc (10-11)	none	50 mg/300 g/day PO
	1	74	Purina chow	1% pCPA 8% suc (day 11) (pair-fed)	none	50 mg/300 g/day PO
(b)	1	106	Purina chow	3.5% Phe 1.0% pCPA 8% suc (day 1)	day 10 → 1.0% pCPA 8% suc	50 mg/300 g/day PO
	1	100	Purina chow	3.5% Phe 1.0% pCPA 8% suc (day 1)	day 6 → 1.0% pCPA 8% suc	50 mg/300 g/day PO
	3	120-131	3.5% Phe 1.0% pCPA 0.125% Asp	3.5% Phe 1.0% pCPA 0.125% Asp**	none	50 mg/300 g/day PO
(c)	1	101	Purina chow	3.5% Phe 1.0% pCPA 8% suc (day 1)	none	50 mg/300 g/day PO
	1	104	Purina chow	3.5% Phe 1.0% pCPA 8% suc (day 2)	day 12 → Purina chow	50 mg/300 g/day PO (until day 12)
(d)	3	85-90	0.025% Asp	1.0% pCPA 0.175% Asp (day 1)	none	50 mg/300 g/day PO
	2	66-74	Purina chow	3.5% Phe 1.0% pCPA 8% suc (~day 40)	none	40mg/300 g/day IP(1) 50mg/300 g/day PO(1)
(e)	2	~60	Purina chow	3.5% Phe 8% suc (~20-40)	none	none
	2	~60	Purina chow	3.5% Phe 0.75% Sessalom (~20-40)	none	none

Continued...

Table 38 Series E, Experiment 2 (Con't.)

GROUP	ANIMALS		DIETS			Ascorbic acid during pregnancy
	Number	Age on gestation day 1*	Before Pregnancy	Test Diet (gestation day begun)	Change during pregnancy	
(f)	2	90, 109	Purina chow	Low Vitamin C (day 1, 2)	none	none

* days
** testdiet started 31-42 days before pregnancy
*** Phe = phenylalanine
pCPA = p-chlorophenylalanine
suc = sucrose
Asp = Aspartame
PO = oral route
IP = intraperitoneal route

Group (b) was composed of those animals which were given test diets supplemented with 1% pCPA 8% sucrose on gestation days 6 to 11. These animals consumed only Purina chow prior to pregnancy. One animal was started on the 1% pCPA 8% sucrose test diet on gestation day 11 and was pair-fed with an animal in group (a). Two additional guinea pigs included in group (b) were initially given a 3.5% Phe 1.0% pCPA 8% sucrose diet on day 1 of gestation but continued poor dietary intake lead to replacement of this test diet with the 1% pCPA 8% sucrose diet on gestation days 6 and 10. When consuming test diets the animals in this group received oral ascorbic acid supplements of 50 mg/300 g/day. Plasma for PA and Tyr estimations was collected on a number of gestation days while the animals were eating the 1.0% pCPA test diets.

Group (c) was included to examine the effect of beginning the 3.5% Phe 1.0% pCPA test diets prior to conception or on days 1 and 2 of pregnancy. Three (3) guinea pigs were given a 3.5% Phe 1.0% pCPA 0.125% Aspartame diet 31 to 42 days before mating occured and these animals were therefore consuming normal quantities of test diet during early pregnancy. These guinea pigs had previously been included in group (d) of Series C, Experiment 5, and were mated at the conclusion of that study. An additional guinea pig was given the 3.5% Phe 1.0% pCPA 8% sucrose diet on day one (1) of gestation after previously eating only Purina chow. The remaining animal in this group was placed on the 3.5% Phe 1.0% pCPA

8% sucrose diet on day 2 of pregnancy; this animal failed to consume even minimally adequate quantities of this test diet and was therefore given Purina chow on gestation day 12. When consuming test diets all animals in this group received ascorbic acid 50 mg/300 g/day orally, and all were bled on a number of occasions.

Group (d) was composed of 3 animals given a 1.0% pCPA 0.175% Asp test diet on day 1 of pregnancy. In order to facilitate acceptance of Asp sweetened test diets these pigs were fed a laboratory prepared diet supplemented only with 0.025% Asp until mating occured. Ascorbic acid (50 mg/300 g/day) was given orally both during the 1.0% pCPA and 0.025% Asp diet periods. Plasma was collected for PA, Phe, and Tyr estimation on gestation days 1, 10, 15 and 19.

Group (e) was composed of 6 pregnant guinea pigs whose day of mating was not detected (or which were inadvertently shipped pregnant by the supplier) and were therefore given test diets in midpregnancy. Two animals were given a 3.5% Phe 1.0% pCPA 8% sucrose test diet and daily ascorbic acid supplement (one, 40 mg/300 g IP, and the other 50 mg/300 g orally). Four (4) additional animals were given commercially prepared diets (Teklad Mills, Madison, Wisconsin) containing 3.5% Phe, 1.4 g/kg of ascorbic acid, and flavored with either 8% sucrose or 0.75 g/kg of Sessalom (a sweet flavoring compound suggested for guinea pigs); these animals were not given additional ascorbic acid supplements. All animals in group (e)

refused to eat their respective test diets and therefore illustrate the effects of near starvation in midpregnancy. Blood was not collected during the test diet period.

Group (f) examined the effect of low vitamin C intake in the absence of Phe and pCPA supplementation. Two (2) animals were given a low vitamin C diet on day one or two of pregnancy and no additional ascorbic acid. This diet was prepared in the laboratory from Purina chow as described above except that no Phe, pCPA, or flavoring was added; diets handled in this manner were found to have approximately 25% of the ascorbic acid content of Purina chow (see Series D, Expt. 1). Phe and Tyr were not estimated in these animals during the low vitamin C diet period.

Results

All animals which continued to consume amino acid supplemented test diets aborted or carried only dead embryos at autopsy. Abortion was detected by the presence of an open vaginal membrane and vaginal bleeding, usually accompanied by passage of recognizable products of conception. Table 39 indicates the gestation days when abortion occured in each group, [one animal in group (c) refused to eat and was therefore killed on gestation day 19 and found to have dead embryos which have been counted as an abortion for analysis]. The results of this study are presented by a consideration of a number of factors which may have contributed

Table 39 Series E, Experiment 2 Gestation day of abortion in each test diet group.

Group	Test Diet (gestation day diet started)	Number of Animals	Gestation Day of Abortion
(a)	3.5% Phe 1.0% pCPA 8% sucrose (day 10-11)	4	16-21
		1	31
		1	41
(b)	1.0% pCPA 8% sucrose (day 6-11)	3	20-29
(c)	3.5% Phe 1.0% pCPA { 8% sucrose or 0.125% Aspartame } (prepregnancy or day 1-2)	3	17-25**
		1	38
	3.5% Phe 1.0% pCPA 8% sucrose (day 2) → Purina →(day 12)	1	none
(d)	1.0% pCPA 0.175% Aspartame (day 1)	3	19
(e)	3.5% Phe±1.0% pCPA { 8% sucrose or 0.75% Sessalom } *	6	2-5 days after test diet begun*
(f)	Low Vitamin C (day 1-2)	2	none

* actual gestation day not known, but estimated day of abortion between 20 and 45 days gestation.

** one animal was killed on day 19 after refusing feeds; only dead embryos were observed.

to this pregnancy wastage.

Diet: Animals which continued to eat diets supplemented with both Phe and pCPA, groups (a) and (c), aborted between gestation days 16 and 41. The food intake of one animal in group (c) became dangerously low and she was therefore given Purina chow on day 12; this animal then progressed well and carried macroscopically normal fetuses to term while consuming the Purina diet. The two animals in group (e) which were initially given the 3.5% Phe 1.0% pCPA diet ate negligible amounts of food (less than 5 g total intake over 3 days), and therefore cannot justifiably be included when assessing the effects of the 3.5% Phe 1.0% pCPA diet on pregnancy (see below).

Animals in group (b) consumed primarily a diet supplemented only with 1.0% pCPA and 8% sucrose; these animals aborted between days 20 and 29 of pregnancy. Two of these guinea pigs were initially given 3.5% Phe 1.0% pCPA 8% sucrose diets but because of poor intake (one ate less than 3 g in 5 days) were switched to the 1.0% pCPA diets on gestation days 6 and 10. The animals in group (d) consumed only the 1.0% pCPA diet during pregnancy and all of these pigs aborted on gestation day 19.

Abortion occured in groups (a), (b), (c), and (d) in guinea pigs maintained on diets containing 1.0% pCPA with or without additional Phe supplement. The animals in group (e) however, aborted within 2 to 5 days of exposure to test diets in spite of negligible pCPA consumption. The intake of pCPA

among the two animals in group (e) given the 3.5% Phe 1.0% pCPA test diet was minimal; the total food intake of these two animals was only 3 g over 4 days for one and 5 g over 3 days for the other (Table 40). The remaining 4 animals in group (e) were not exposed to pCPA containing test diets although they too aborted; their dietary intake however, was also minimal (see below).

The two animals fed the unsupplemented laboratory prepared low ascorbic acid diet, group (f), carried their pregnancies to near term when they were sacrificed and found to have normal living fetuses (Table 39).

Dietary Intake: The daily food intake of each animal in group (a) is illustrated in Figure 18. It is clear that all of the animals dramatically reduced dietary intake when first presented with the test diet. The animal which maintained the pregnancy to 41 days had the best intake of test diet, at least until a few days prior to abortion. The three guinea pigs which aborted earliest, days 16 to 18, were the only animals in this group not to reach pre-test diet intake levels on a single day prior to abortion. Although the animals aborting on days 21 and 31 generally had better intakes than those aborting earlier, neither animal consistently maintained a normal food consumption.

Two of the animals in group (b) maintained nearly normal intakes of the 1.0% pCPA test diet, yet aborted on days 25 and 29 (Figure 19). Both of these guinea pigs however, were

Table 40 Series E, Experiment 2, Group (e). Daily food intake (g/24 hours) for the 3 days prior to the test diet period and for the test diet period to the day of abortion.

Animal Number	Days on Test Diet								
	-3	-2	-1	+1	+2	+3	+4	+5	Mean Test Diet
26	--	--	--	2	0	1	0*	--	0.75
6A	--	--	--	5	0	0	0	0*	1.0
12A	33	35	35	3	0	1	1	-*	1.0
13A	35	30	32	3	0*	--	--	--	1.5
18A	36	32	33	2	0	8	16*	--	6.5
19A	35	36	36	1	0	0	0*	--	0.25

* Day of abortion

Figure 18. Series E, Experiment 2. Group (a). Daily food intake during pregnancy of each animal. Closed circles: Purina chow; open triangles: 3.5% Phe 1.0% pCPA 8% sucrose test diet. Ⓐ indicates gestation day of abortion.

Figure 18

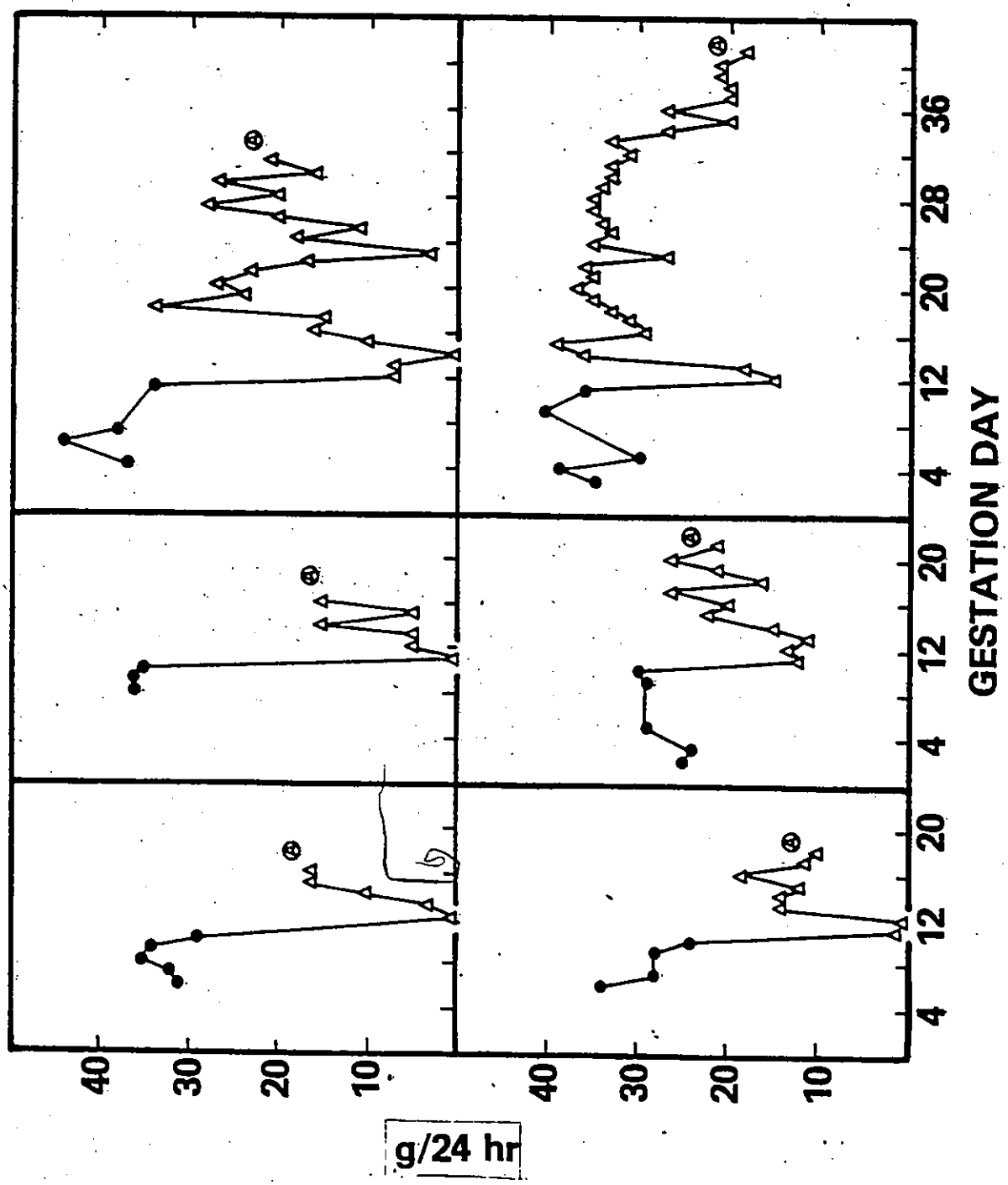
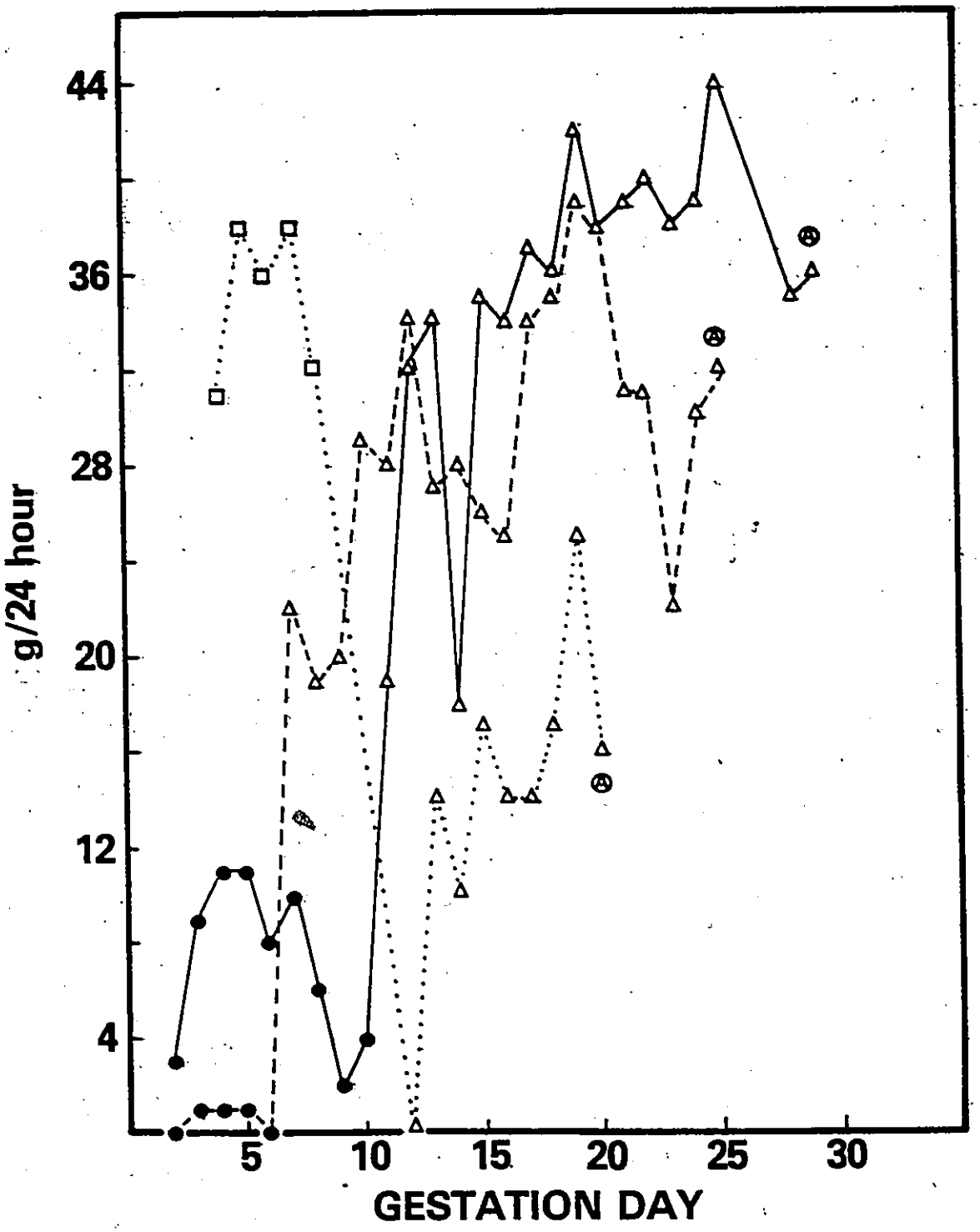


Figure 19. Series E, Experiment 2. Group (b). Daily food intake for each animal during pregnancy. Closed circles: 3.5% Phe 1.0% pCPA 8% sucrose test diet. Open squares: Purina chow. Open triangles: 1% pCPA 8% sucrose test diet. Ⓐ indicates gestation day of abortion.

Figure 19

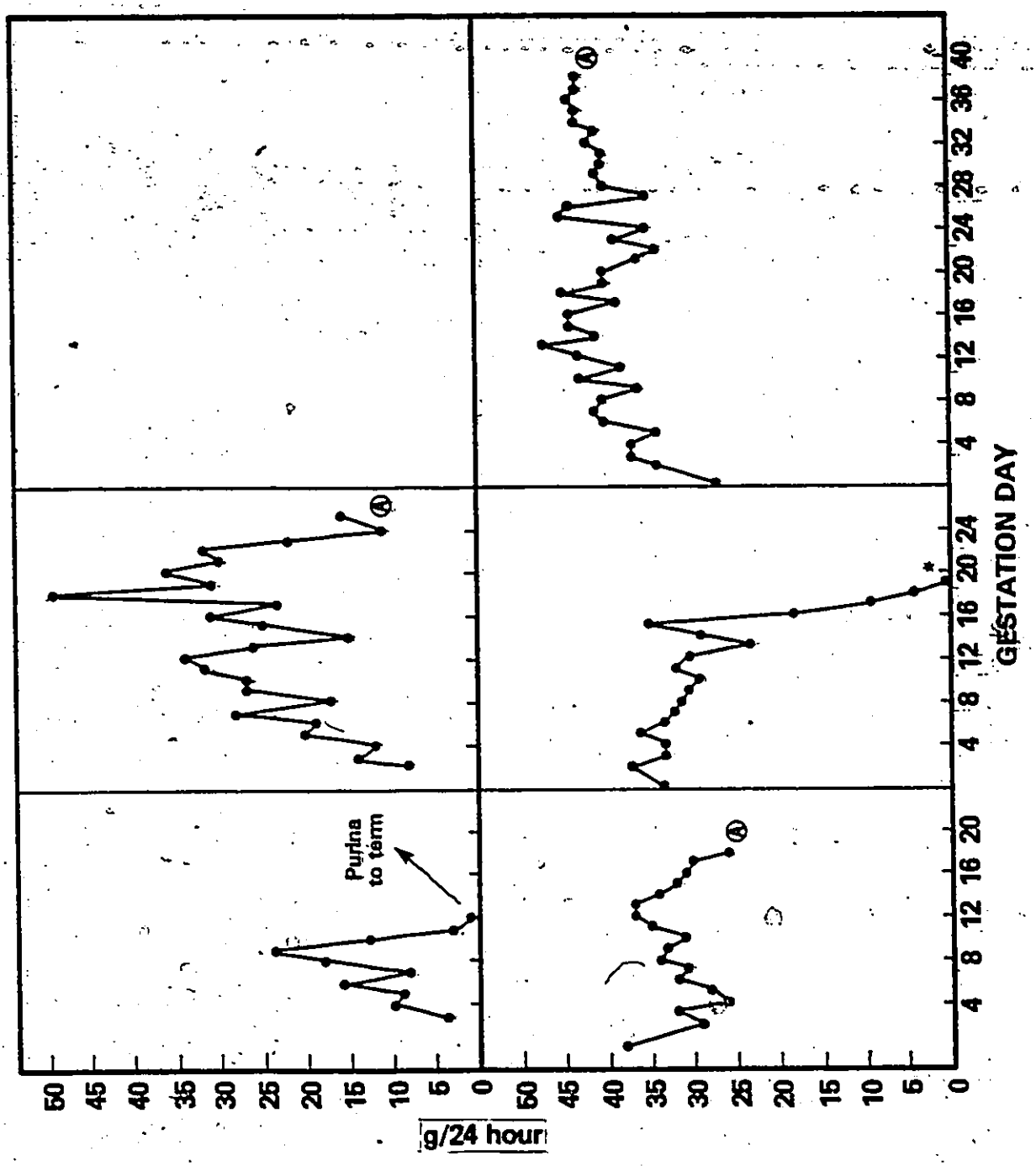


initially given the 3.5% Phe 1.0% pCPA test diet and had very low dietary intakes early in pregnancy. The third animal in this group had a subnormal intake throughout the test diet period and aborted on gestation day 20, 9 days after the 1.0% pCPA diet was presented. Food intake of this animal was normal during the first 10 days of gestation, when pair-feeding with an animal in group (a) began. The corresponding guinea pig in group (a) aborted on gestation day 16.

In contrast to groups (a) and (b), three of the animals in group (c) were accustomed to the 3.5% Phe 1.0% pCPA test diet prior to conception and were therefore consuming normal quantities of test diet during early pregnancy. Daily intake of these animals is illustrated in the lower 3 panels of Figure 20. Two of these animals consumed normal quantities of test diet initially and then consumption declined prior to abortion (sacrifice in one animal which was undoubtably about to die). One animal however, continued to eat normal quantities of test diet throughout pregnancy yet aborted on day 38. This normal food consumption was reflected by a steady increase in body weight during pregnancy (gain of 88 g). Changes in body weight among the guinea pigs in all groups were roughly directly related to dietary intake. The two remaining animals in group (c) were given the test diets on days 1 or 2 of pregnancy (upper panels Figure 20) and both exhibited poor intake during the initial days on the test diet. One animal subsequently refused to eat the 3.5% Phe 1.0% pCPA diet and

Figure 20. Series E, Experiment 2. Group (c). Daily food intake of each animal during pregnancy. Closed circles: 3.5% Phe 1.0% pCPA test diet. Ⓐ indicates day of abortion. * Animal sacrificed and dead embryos observed.

Figure 20



was therefore given Purina chow from gestation day 12 until term. In spite of a very low food intake early in pregnancy this animal carried normal fetuses to term. The remaining animal given test diet early in gestation consumed variable quantities of the test diet and aborted on day 25.

With the exception of the first day on the 1.0% pCPA test diet, the animals in group (d) maintained approximately normal dietary intakes, yet all aborted on gestation day 19 (Figure 21). All of these animals gained weight during the 19 days of pregnancy (average 26 g).

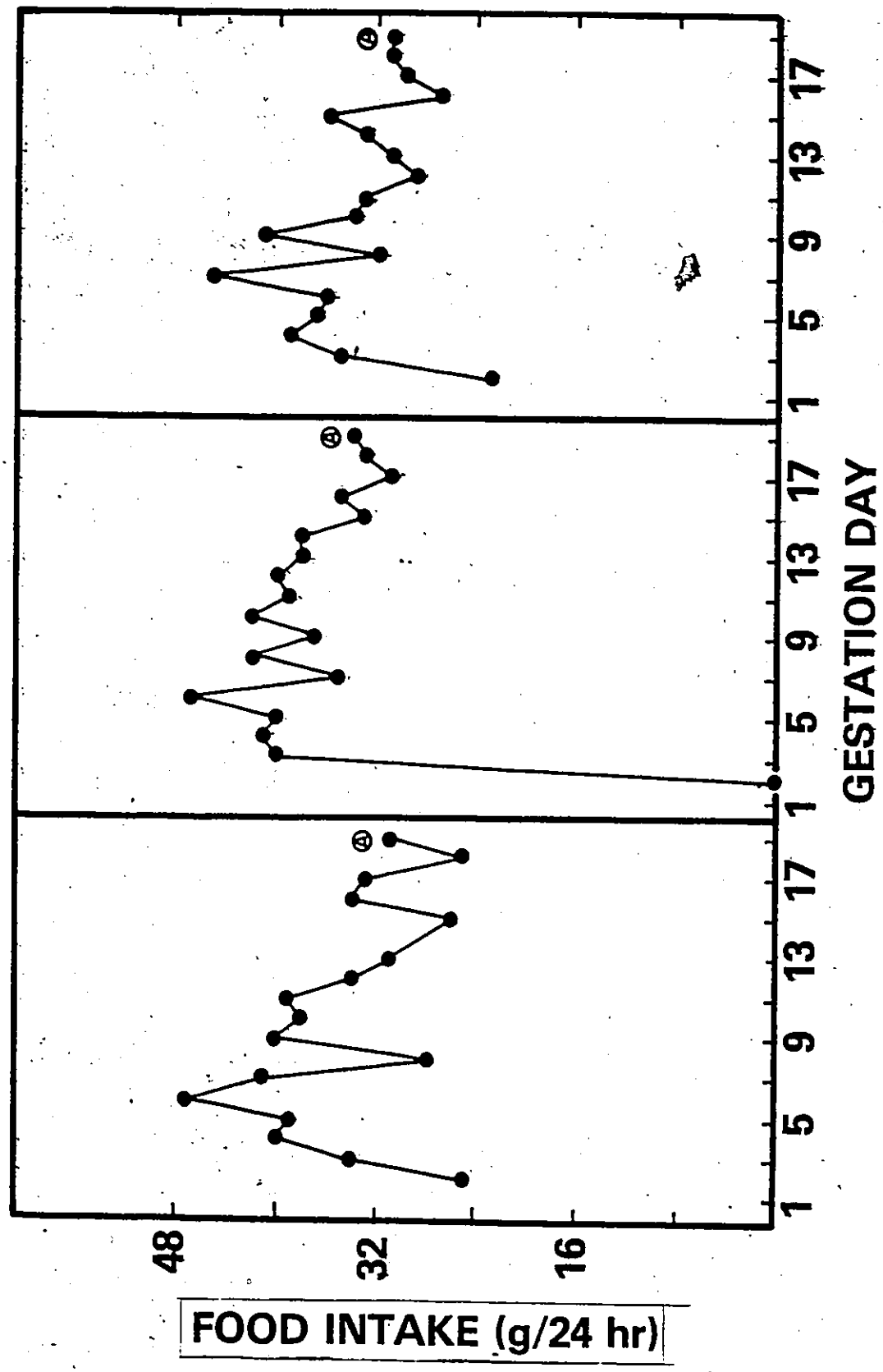
Animals in group (e) were given test diets during midgestation and their daily food intake was extremely poor (Table 40). Only one guinea pig averaged over 2 g/24 hours (6.5 g/24 hour) and even this is well below a normal daily intake (greater than 30 g/24 hour). By 5 days after the test diets were presented to these animals all had aborted, illustrating that near starvation in midgestation is not compatible with the maintainance of pregnancy in the guinea pig.

Both animals in group (f) consumed normal quantities of the low vitamin C diet and had normal fetuses near term.

Critical Period: Implantation of the guinea pig embryo occurs about day 6 to 7 of pregnancy and it is a potentially vulnerable event. Animals were given 3.5% Phe 1% pCPA test diets both before implantation, group (c), and after implantation was complete, group (a). Females in both groups however,

Figure 21. Series E, Experiment 2. Group (d). Daily food intake of each animal during pregnancy. Ⓐ indicates day of abortion.

Figure 21



aborted at similar times during pregnancy (Table 39). Guinea pigs were also fed 1% pCPA supplemented test diets prior to implantation, group (d), and after implantation, group (b). All the animals in group (d) aborted on gestation day 19 (Table 39). The animals in group (b) aborted between 20 and 29 days. One of these animals was given the 1.0% pCPA test diet on day 6 after refusing the 3.5% Phe 1.0% pCPA diet; this pregnancy was lost on gestation day 25 (Figure 19). An additional animal aborted on day 29 after being switched from the 3.5% Phe 1.0% pCPA diet to 1.0% pCPA on gestation day 10.

Animals in group (e) illustrate that the postimplantation period, calculated to be from days 20 to 45 in these particular guinea pigs, was extremely sensitive to the combination of very low dietary intake and Phe supplemented test diets.

PA, Phe, Tyr Concentration: Plasma for estimation of PA, Phe, and Tyr was collected during pregnancy from animals in groups (a), (b), (c), and (d) and the results are summarized in Figure 22 and Table 41. One animal in group (a) which aborted on day 21, had baseline plasma Tyr levels between 14 and 18 mg/100 ml and was therefore excluded from the PA, Phe, and Tyr data presented in this experiment (see Appendix B). Animals consuming the 3.5% Phe 1.0% pCPA test diets, groups (a) and (c), had PA levels averaging about 21 mg/100 ml and Phe levels averaging from 9 to 13 mg/100 ml. In contrast to these data, animals fed the 1.0% pCPA test diets had average PA levels of 11 to 13 mg % and Phe levels which were

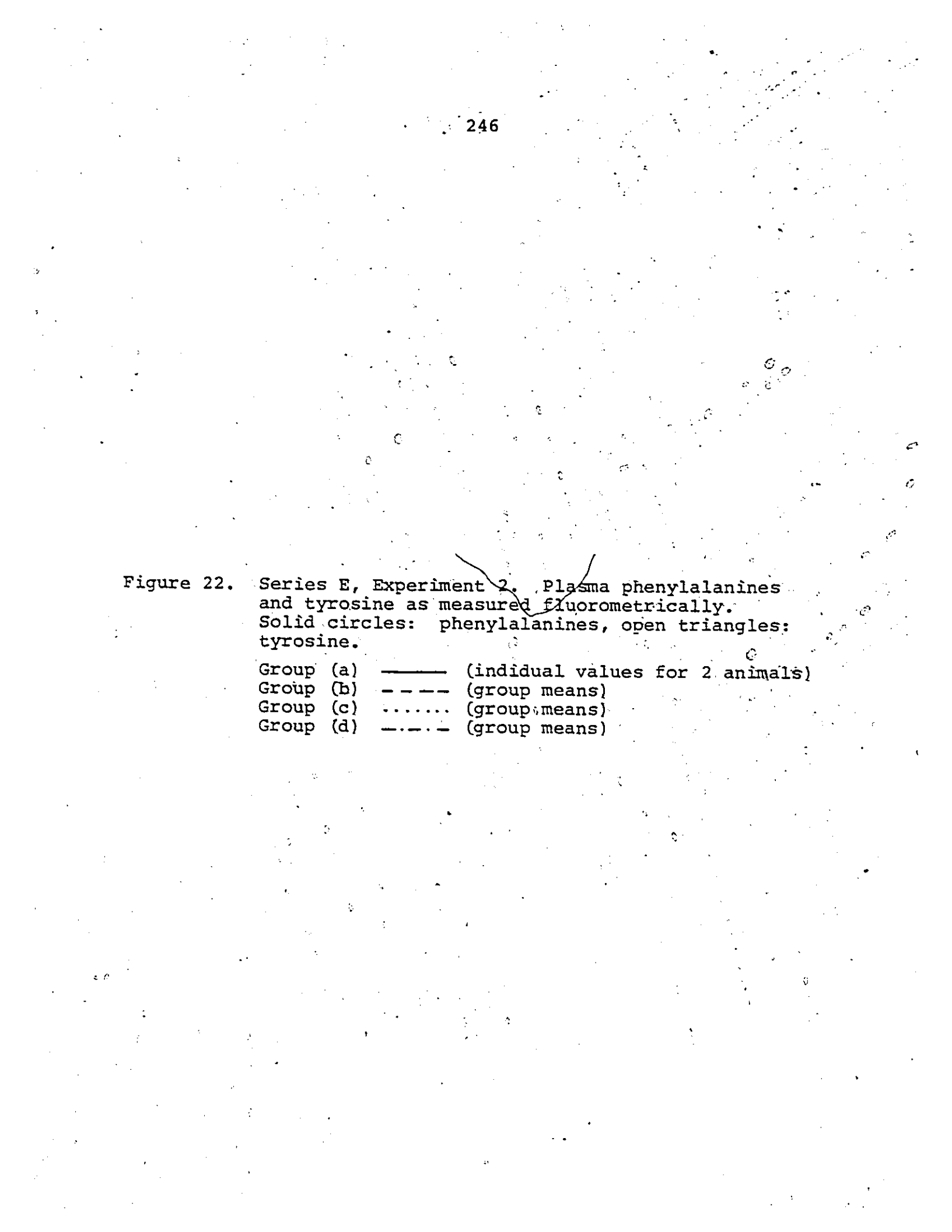


Figure 22. Series E, Experiment 2. Plasma phenylalanines and tyrosine as measured fluorometrically. Solid circles: phenylalanines, open triangles: tyrosine.

Group (a) ——— (indidual values for 2 animals)
Group (b) – – – – (group means)
Group (c) ..... (group means)
Group (d) —.—.— (group means)

Figure 22

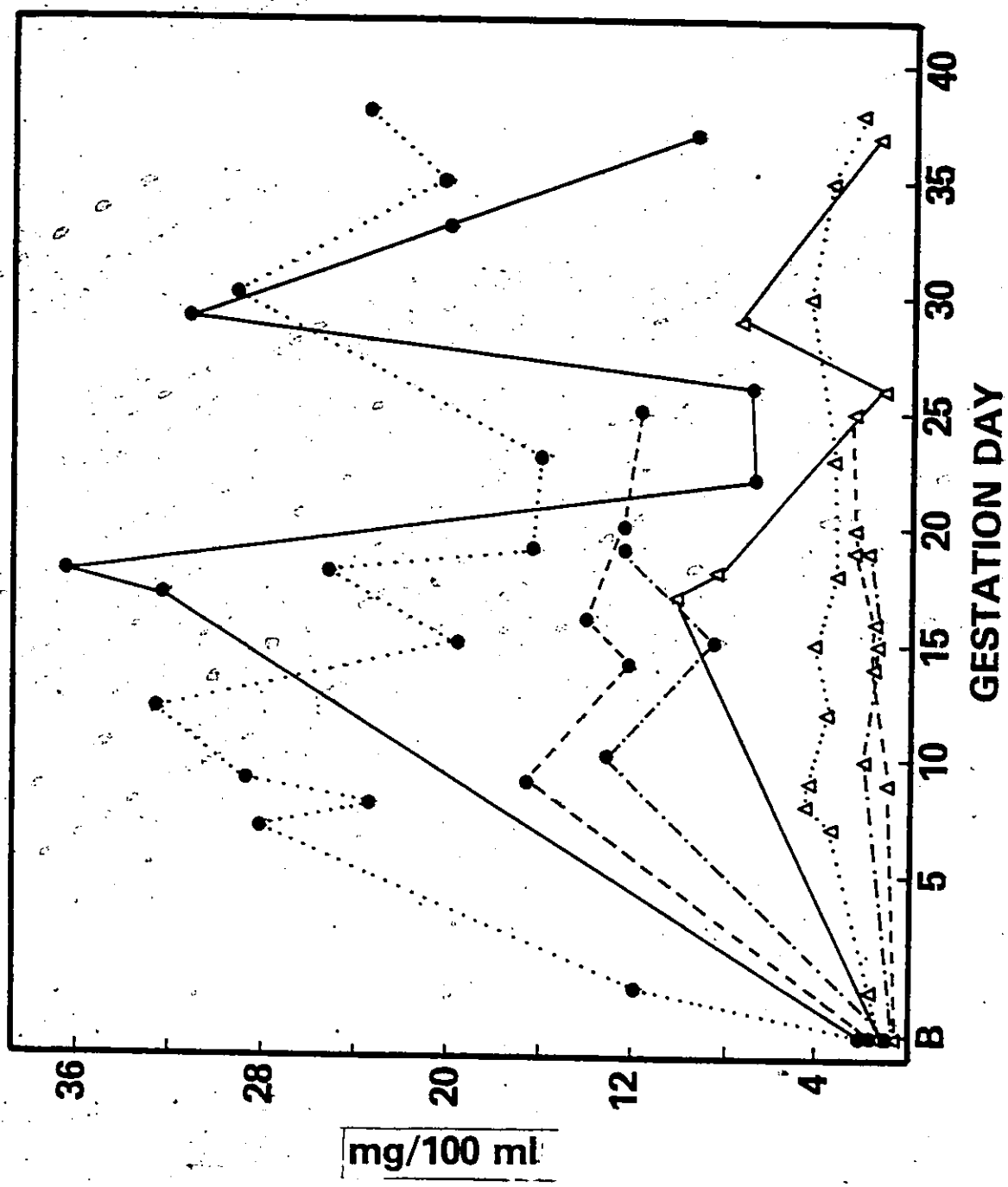


Table 41 Series E, Experiment 2. Plasma phenylalanines and tyrosine (mg/100 ml, mean ±S.D.). For calculations, paper values below limits of detection taken as 3 mg/100 ml and 2.0 mg/100 ml for Phe and Tyr, respectively.

	Group		Prediet	GESTATION DAY 1	7-10	12-14	15-16	17	18-20	22-23	25-26	29-30	33-35	37-38	Overall (Test diet)
(a)	Fluoro**	N*	5					1	1	1	1	1	1	1	7
		PA	2.08±1.14					32.4	38.2	6.8	7.0	31.3	20.0	9.5	20.74±13.31
		Tyr	1.12±0.68					10.2	8.1		1.2	7.5		1.5	5.70±4.10
	Paper	N	1					1						1	2
		Phe	3					16						3	9.5±9.2
		Tyr	2					2						2	2.0±0
(b)	Fluoro	N	1		1	1	1		2		1				6
		PA	1.6		1.6	12.2	14.1		12.5±1.0		11.8				13.28±1.86
		Tyr	0.6		1.0	1.6	1.6		2.4±2.55		2.6				1.93±1.30
	Paper	N			1	1	1		1		1				5
		Phe			3	4	3		3		3				3.2±0.4
		Tyr			2	2	2		2		2				2.0±0
(c)	Fluoro	N	5	3	4	1	3		4	1		1	1	1	19
		PA	1.22±0.23	11.93±6.14	27.30±4.58	32.6	19.73±7.74		20.82±12.12	16.1		29.2	20.4	23.6	21.55±8.62
		Tyr	1.16±0.09	1.73±0.49	4.18±0.61	3.7	4.07±2.20		2.85±	3.4		4.4	3.6	2.2	3.31±127

Continued...

Table 41 Series E, Experiment 2.
(Con't.)

	Group		Prediet	GESTATION DAY 1	7-10	12-14	15-16	17	18-20	22-23	25-26	29-30	33-35	37-38	Overall (Test diet)
(c)	Paper	N	5	3	4	1	1		4	1		1	1	1	17
		Phe	3.0±0	3.7±0.6	14.0±5.9	24	16		13.2±7.6	12		18	14	16	12.9±6.7
		Tyr	2.0±0	2.0±0	3.2±1.5	2	5		2.0±0	2		2	2	2	2.5±1.1
	Fluoro	N	3		3		3		3						9
		PA	0.90±0.10		13.17±1.99		8.47±1.42		12.53±1.95						11.39±2.71
		Tyr	0.67±0.12		2.00±0.35		1.53±0.32		1.93±0.12						1.82±0.33
(d)	Paper	N	3		3		3		3						9
		Phe	3.0±0		3.0±0		3.0±0		3.0±0						3.0±0
		Tyr	2.0±0		2.0±0		2.0±0		2.0±0						2.0±0

*N = number of samples, PA = total phenylalanines, Phe = phenylalanine, Tyr = tyrosine
** Fluoro = fluorometric assay, Paper = paper chromatographic assay.

generally undetectable by the paper chromatographic method [groups (b) and (d), Table 41]. Plasma Phe as measured by paper chromatography, was significantly greater in groups (c) than in groups (b) ($t = 3.210$, $df = 20$, $p<.01$, two-tailed) and (d) ($t = 4.431$, $df = 24$, $p<.001$, two-tailed). Plasma PA tended to be higher in the 3.5% Phe 1.0% pCPA groups and group (c) was significantly greater than group (d) ($t = 3.428$, $df = 26$, $p<.01$, two-tailed) but only marginally greater than group (b) ($t = 2.301$, $df = 23$, $p<.05$, two-tailed). Unpaired t-tests did not reveal any additional statistically significant differences between groups with respect to plasma Phe and PA. Tyr levels were higher in the 3.5% Phe 1.0% pCPA groups. Plasma Tyr, as measured fluorometrically, was significantly greater in both groups (a) and (c) when compared to group (d) [(d) vs (a), $t = 2.921$, $df = 12$, $p<.02$, two-tailed; (d) vs (c), $t = 3.406$, $df = 26$, $p<.01$, two-tailed], although a significant difference was not found between group (b) and (a) ($t = 2.146$, $df = 9$). Between the two 3.5% Phe 1.0% pCPA diet groups, Tyr was marginally greater in group (a) compared to (c) ($t = 2.276$, $df = 22$, $p<.05$, two-tailed). Other t-tests of the plasma Tyr data were not statistically significant.

No blood was analyzed for amino acids during the test diet period in groups (e) and (f), although it is unlikely that either group had significant hyperphe or hypertyrosinemia.

There were higher Phe, PA, and Tyr levels in the guinea pigs fed the 3.5% Phe 1.0% pCPA diets, yet abortion often

occured later in these animals. From these data, it is not possible to determine the relative importance of elevated amino acid levels in the etiology of guinea pig abortion except to note that abortion in some cases occured in the absence of both hyperphe and hypertyrosinemia.

Comparison of the time when abortion occured (Table 39) and the PA, Phe, and Tyr blood concentrations during those periods (Table 41, Figure 22) does not reveal any obvious relationship, although the data are limited.

Urine $FeCl_3$ Tests: Three urine samples from one animal in group (a) were tested for phenylketones with $FeCl_3$ and all were positive. An additional 3 samples from the animal in group (a) with the pre-test diet elevated Tyr were collected during the test diet period and also found to be positive. One of two samples in group (b) and both samples in group (d) were positive. No other urine samples were available for $FeCl_3$ testing. These limited data indicate that pregnant guinea pigs fed test diets supplemented with 1% pCPA with and without Phe have positive urinary $FeCl_3$ tests, indicating excessive phenylketone excretion.

Series E, Experiment 3

Introduction

The results of Series E, Experiment 2 indicated that pregnant guinea pigs fed test diets containing 1% pCPA with or without 3.5% Phe aborted between gestation days 16 and 41. The two factors which were found to most clearly predispose to pregnancy loss were the presence of pCPA in the diet and inadequate dietary intake. Neither hyperphe nor vitamin C deficiency appeared to be major factors leading to pregnancy wastage. The time period around implantation of the embryo was not found to be disrupted by the dietary treatments, whereas midpregnancy was very sensitive to severe maternal undernutrition. This experiment also demonstrated that feeding pregnant guinea pigs the 3.5% Phe 1.0% pCPA diet produced hyperphe similar to that found in nonpregnant animals fed this diet (cf Tables 12, 13, 18, 19, 24, 33, Figures 5, 8).

It is important to further delineate the effects of hyperphe and pCPA on early guinea pig pregnancy since there may be an increased risk of congenital malformation and abortion associated with maternal hyperphe in humans. In the following experiment guinea pigs were fed diets supplemented with Phe and pCPA from the first day of pregnancy. To permit study of embryonic material the animals were killed

on gestation day 17, prior to the time when abortion had been observed to occur. The embryos were examined both macroscopically and microscopically and in addition, yolk sac-amniotic fluid was collected for estimation of Phe, PA, and Tyr. Maternal plasma PA, Phe, and Tyr, as well as maternal hepatic Phe hydroxylase activity and ascorbic acid content, were also measured. Five groups were included: the 3.5% Phe 1.0% pCPA experimental group and four control groups. A 0.175% Asp supplement was added to Phe and pCPA supplemented test diets in order to maximize maternal food intake and nutrition (Series C, Experiment 5).

Methods

All guinea pigs were Hartley strain and obtained from M. & L. Farms. Females ranged from 91 to 113 days of age on gestation day 1 and males between 130 and 440 days of age during the mating period. All females used in this experiment were received in one shipment from the supplier, and none of these females had been pregnant prior to the experiment. Females were housed 2-3 per cage as described above until isolated when mating was detected.

All Phe and pCPA supplemented test diets were sweetened with Aspartame. In order to minimize the decline in food intake associated with Phe and pCPA test diets, the animals were allowed to become accustomed to the sweetness of Aspartame prior to pregnancy. All females were fed a laboratory prepared diet supplemented only with 0.025% Aspartame

from the age of 74 days until mating was detected at which time each animal was given the appropriate test diet. (see below) All animals gained weight steadily while consuming the 0.025% Asp diet. These animals also received daily oral ascorbic acid, 50 mg/300 g, as described above.

Females were placed with potent males and checked twice daily for indications of mating. The day a positive vaginal smear was detected was designated day 1 of gestation. Within 18 hours of such a smear, each female was bled, isolated and given the diet appropriate to the assigned group. Thirty (30) females were assigned to this study, but some were subsequently excluded (see below). Nineteen (19) females were randomly assigned on the basis of mating sequence to one of the five (5) test diet groups. Three additional animals were subsequently assigned (non randomly) to study groups in order to increase the numbers in groups which had lost subjects, [animals were excluded from the study if they were found to have high "baseline" plasma Tyr (3 animals, see Appendix B), if they refused to eat the test diet (3 animals), or if they were not killed on gestation day 17 (5 animals, see below)]. The following 5 diet groups were included:

Group (a): 3.5% Phe 1.0% pCPA 0.175% Asp test diet, 4 females.

Group (b): 3.5% Phe 0.175% Asp test diet, 4 females.

Group (c): 1.0% pCPA 0.175% Asp test diet, 4 females.

Group (d): 0.025% Asp test diet, 4 females. A

larger supplement of Asp was not used because the excessive sweetness in the absence of Phe and pCPA supplements may have lead to inadequate food intake.

Group (e): Purina chow, 3 females.

Animals in groups (a), (b), (c), and (d), but not (e), received daily oral ascorbic acid, 50 mg/300 g, from gestation day 1 until sacrificed. Heparinized plasma for Phe, PA and Tyr measurement was collected from the saphenous vein from all animals between about 1000 and 1400 hours on gestation days 1 (within 18 hours of pregnancy detection), 10, 15, and 17. The samples were assayed in duplicate, fluorometrically and by paper chromatography. On gestation day 17 additional plasma for analysis on the Beckman Amino Acid Analyzer was collected from 2 animals in groups (a), (d) and (e) and from 3 animals in groups (b) and (c). Body weight, food intake and signs of abortion were monitored daily in all animals. $FeCl_3$ tests for phenylketones were performed when fresh urine was available.

On gestation day 17 the females were killed by decapitation and the uteri quickly removed and placed in a dish of 0.85% saline. The maternal liver was then promptly removed blotted dry, weighed, and frozen in solid CO_2. The uteri were carefully examined under a dissecting microscope and the number and location of implantation sites recorded. The uterine wall was cut and the decidual masses containing the embryos shelled out. At this point it was usually

possible to determine if an embryo was living by the presence of an active heart beat and/or blood in the vitelline and embryonic vessels. When possible, prior to rupture of the yolk sac membrane, yolk sac fluid was collected with a 50 µl Hamilton microsyringe for later analysis of PA, Phe, and Tyr. Embryos were examined, photographed and the number of somites counted. The embryos were fixed in Bouin's fluid, embedded in paraffin, cut serially at 10 µm, and stained with hematoxylin and eosin. Except for group (e), which was used to establish a normal baseline, the slides were coded by someone unfamiliar with the details of the experiment and subsequently examined in a blind fashion. Histologic abnormalities were also photographed.

Within 24 hours of collection, the maternal livers were homogenized in cold 0.1M phosphate buffer (pH 7.0) and assayed in duplicate for Phe hydroxylase as described above. Four days after death, maternal liver was homogenized in cold 5% TCA and assayed in triplicate for ascorbic acid as described above.

Results

The original design of this study called for sacrifice on gestation day 25. The first 3 animals assigned to the 1.0% pCPA group however, aborted on day 19 and the decision was therefore made to kill subsequent animals on day 17. These 3 animals have been reported in Series E, Experiment 2 as

group (d). Only those animals which were killed on gestation day 17 are included in the present study making a total of 19 distributed among the five groups. Of the 19 animals included in the study one in group (a), 3.5% Phe 1.0% pCPA diet, aborted on day 17. Embryos were collected from all other females on gestation day 17.

Embryos. Whole embryos were examined under the dissecting microscope when the pregnant females were sacrificed, and again following fixation. Each embryo was then studied histologically with all but those in group (e) done so that the author was not aware of the group from which the embryo came. For purposes of statistical analysis the following definitions were used. If an embryo was found to have a beating heart it was designated as living regardless of what was later discovered histologically. If no heart beat or flowing blood was visible in the unfixed embryo, and there were areas of necrosis histologically, then the embryo was classified as dead; however, if there was no necrosis then it was scored as living. When no recognizable embryonic tissue could be found either macroscopically or microscopically, then the "embryo" was designated as dead. Embryos classified as abnormal included dead embryos, live embryos with histological evidence of tissue necrosis, and living but malformed embryos. Embryos with developmental delay were designated as retarded which was defined as follows. Embryos were considered to be retarded if at least two organ systems were

developmentally behind the range observed in the Purina control [group (e)] embryos; such retarded embryos could be either dead or living, as defined above. When an embryo could not be identified, that implantation site was excluded from the retarded/non retarded data. The somite count was not included as a criterion for retardation since those data were analyzed independently.

Table 42 summarizes the data for embryonic mortality, abnormality and retardation. Since each pregnant guinea pig, rather than the individual embryos, was the independant sampling unit, litter scores were generated for each of these variables (Haseman and Hogan 1975; Weil 1970). Live, normal, or unretarded embryos were scored as 2.00 while dead, abnormal, or retarded embryos were scored as 1.00; the mean of these values for each variable in each litter equalled the litter score for that variable (Table 42). Table 42 also shows the number of implantations in each group. One-way ANOVA indicated that there were no significant differences among the five groups with respect to the number of implantation sites ($F = 1.009$, $df = 4, 14$) confirming that implantation was not affected by the maternal treatments (Series E, Experiment 2). As noted above, in a number of implantations, it was not possible to identify recognizable embryonic tissue in spite of the presence of maternal and embryonic decidual tissues of macroscopically normal size. Such implantations were observed only in groups (a) and (c) and are shown in

Table 42 Series E, Experiment 3. Number of implantations, somites, live, abnormal, and retarded embryos plus the litter scores used for statistical analysis.

Group	Female Number	Number of Implantations	Mean number of Somites	Live Embryos** Number	Live Embryos** Litter Score***	Abnormal Embryos** Number	Abnormal Embryos** Litter Score***	Retarded Embryos** Number	Retarded Embryos** Litter Score***
(a)	84A	4	16.2	4	2.00	1	1.75	0	2.00
3.5% Phe	86A*	0*	---*	0*	1.00	-*	1.00	-*	----
1.0% pCPA	10B	3	20.3	3	2.00	1	1.67	0	2.00
	14B	3+	0	0	1.00	3	1.00	-	----
Mean or Total	4	10	12.2	7/10	1.50	5/10	1.36	0/7	2.00
	88A	4	18.5	4	2.00	2	1.50	2	1.50
(b)	3B	1	15	1	2.00	0	2.00	0	2.00
3.5% Phe	8B	5	3.0	5	2.00	4	1.20	4	1.25
	12B	5	16.8	5	2.00	1	1.80	0	2.00
Mean or Total	4	15	13.3	15/15	2.00	7/15	1.62	6/15	1.69
	89A	3+	0	0	1.00	3	1.00	-	----
(c)	90A	4	6.8	4	2.00	0	2.00	4	1.00
1.0% pCPA	7B	5	0	0	1.00	5	1.00	4/4	1.00
	9B	4(1+)	6.0	1	1.25	3	1.25	3/3	1.00
Mean or Total	4	16	3.2	5/16	1.31	11/16	1.31	11/11	1.00
	92A	5	19.5	5	2.00	0	2.00	0	2.00
(d)	93A	3	17.0	3	2.00	0	2.00	0	2.00
0.025% Asp	94A	4	18.0	4	2.00	0	2.00	1	1.75
	95A	5	18.0	5	2.00	2	1.60	0	2.00
Mean or Total	4	17	18.1	17/17	2.00	2/17	1.90	1/17	1.94
(e)	83A	3	18.7	3	2.00	0	2.00	0	2.00
Purina	1B	4	15.8	4	2.00	0	2.00	0	2.00
	2B	4	14.7	4	2.00	0	2.00	0	2.00
Mean or Total	3	11	16.4	11/11	2.00	0/11	2.00	0/11	2.00

* Aborted day 17

\+ Although the decidual masses were grossly normal, no embryos could be detected.

** See text for definitions

*** Litter score: mean score for each litter; 2.00 = Live, normal, or not retarded; 1.00 = Dead, abnormal or retarded

Tables 42 and 43 (see below).

Groups (a) and (c) were also the only groups found to have dead embryos (Table 42). Figure 23 illustrates the percentage of embryos which were scored as living, abnormal or retarded in each of the five groups. ANOVA of the embryonic mortality data, using the litter scores, revealed a significant effect for the test diet group ($F = 3.565$, df = 4, 14, $p = 0.0332$), although a Student-Newman-Keuls multiple range comparison could not detect the individual difference between the groups. It is clear however, that only the groups consuming diets containing pCPA had dead embryos, 30% in the 3.5% Phe 1.0% pCPA group, and 69% in the 1.0% pCPA group (Figure 23, Table 42).

Abnormal embryos were found in all groups except (e), but only groups (a), (b) and (c) had relatively high rates of embryonic abnormality (Figure 23, Table 42). ANOVA of the abnormality data approached, but did not reach statistical significance ($F = 2.953$, df 4, 14, $p = .0581$), and the less sensitive Student-Newman-Keuls comparison was also insignificant at the 0.05 level. It is of note that ANOVA done on the abnormality data using the individual embryos, rather than the litter scores, showed a highly significant group effect ($F = 6.051$, df = 4, 63, $p = 0.0003$). Table 43 categorizes the abnormality data into a number of subgroups including embryos found to be grossly malformed and malformed but alive. All but 2 embryos classified as abnormal were

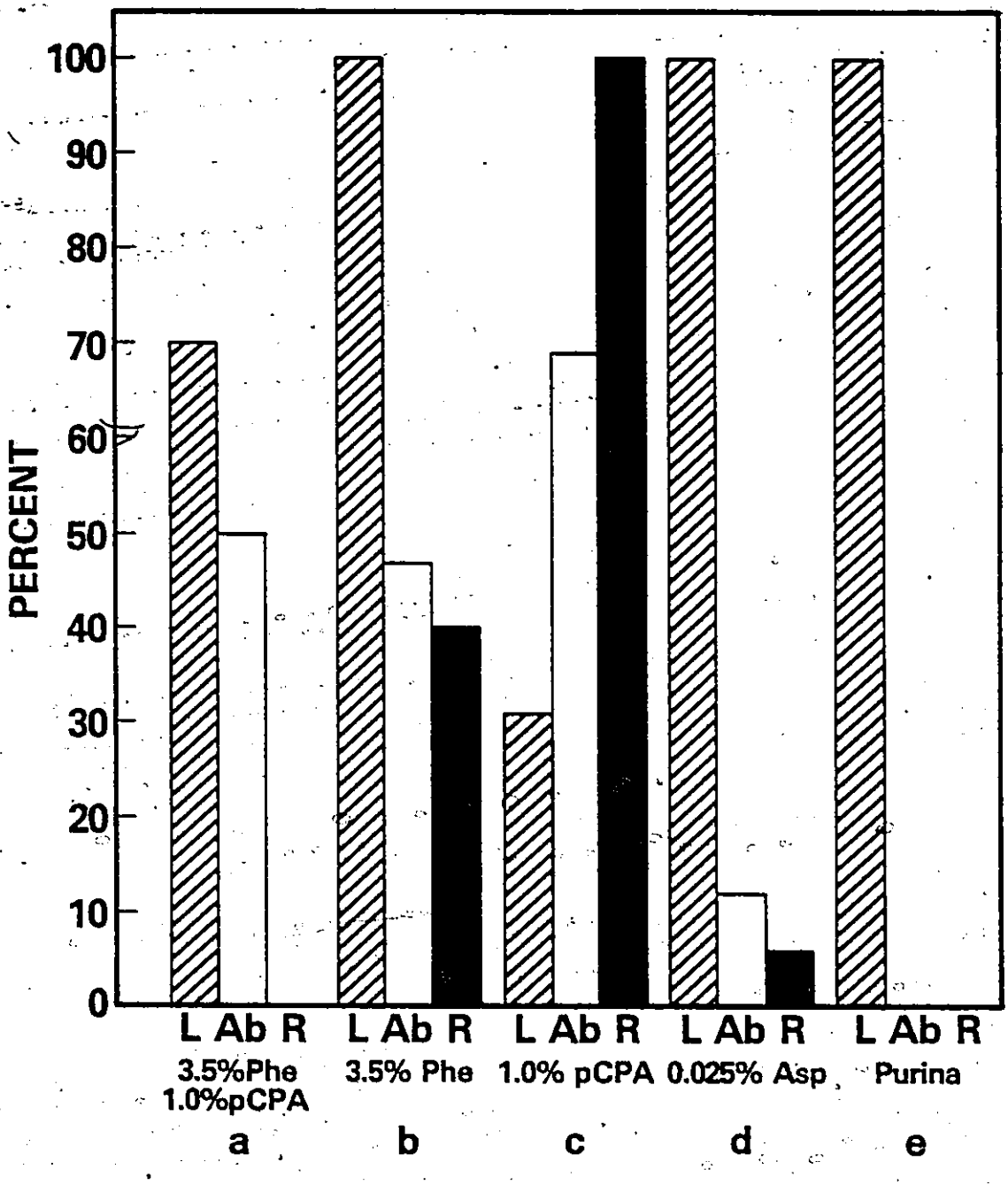


Figure 23. Series E, Experiment 3. Percentage of implantations which were living (L), abnormal (Ab), and retarded (R) in each test diet group.

Table 43 Series E, Experiment 3. Number of abnormal embryos malformed, with local necrosis and number live malformed.

Group	Female #	Number of Implantations	Number Abnormal*	Number Malformed	Number with only Local Necrosis	Living and malformed/ # Live
(a) 3.5% Phe 1.0% pCPA	84A	4	1	1	0	1
	86A**	-	-	-	-	-
	10B	3	1	1	0	1
	14B	3	3	3***	0	0
Total		10	5/10	5/10	0	2/7
(b) 3.5% Phe	88A	4	2	2	0	2
	3B	1	0	0	0	0
	8B	5	4	4	0	4
	12B	5	1	0	1	0
Total		15	7/15	6/15	1/15	6/15
(c) 1.0% pCPA	89A	3	3	3***	0	0
	90A	4	0	0	0	0
	7B	5	5	5	0	0
	9B	4	3	3 (1***)	0	0
Total		16	11/16	11/16	0/16	0/5
(d) 0.025% Asp	92A	5	0	0	0	0
	93A	3	0	0	0	0
	94A	4	0	0	0	0
	95A	5	2	1	1	1
Total		17	2/17	1/17	1/17	1/17

Continued...

Table 43 (Con't.) Series E, Experiment 3

Group	Female #	Number of Implantations	Number Abnormal*	Number Malformed	Number with only Local Necrosis	Living and malformed/ # Live
Purina (e)	83A	3	0	0	0	0
	1B	4	0	0	0	0
	2B	4	0	0	0	0
Total		11	0/11	0/11	0/11	0/11

* see text for definition

** Abortion day 17

*** when no recognizable embryonic tissue was found, that implantation scored as malformed and dead.

indeed malformed, and in groups (a) and (b) many of these malformed embryos were also alive. The types of malformations observed included completely bizzare embryos in which only the germ layers could be identified, overgrown open neural tubes, extra or absent otic vesicles, enlarged pericardial cavities, and abnormalities of the aortic arches (Figures 24 to 26). Many of these malformed embryos also had local areas of necrotic tissue, often in the neuroectoderm.

It is of note that the abnormality observed in the single malformed embryo in group (d), 0.025% Asp diet, consisted of a posterior neural tube which had two independant neural canals. A number of workers have reported this type of neural tube cavitation in both chick and man and have suggested that this is a normal phenomenon in tailless vertebrates (Criley 1969; Hughes and Freeman 1974). The guinea pig is, of course, tailless. Such multiple cavitation however, was not observed in any of the Purina control embryos, (or in any other experimental group), and was therefore counted as a malformation.

No retarded embryos were found in groups (a) and (e), whereas all embryos were retarded in the 1.0% pCPA group and all the malformed embryos in groups (b), 3.5% Phe test diet, were also retarded. Only one retarded embryo was found in the 0.025% Asp group. Two litters in the 3.5% Phe 1.0% pCPA group could not be assessed for the presence of retarded embryos (Table 42) and it is therefore possible

Figure 24

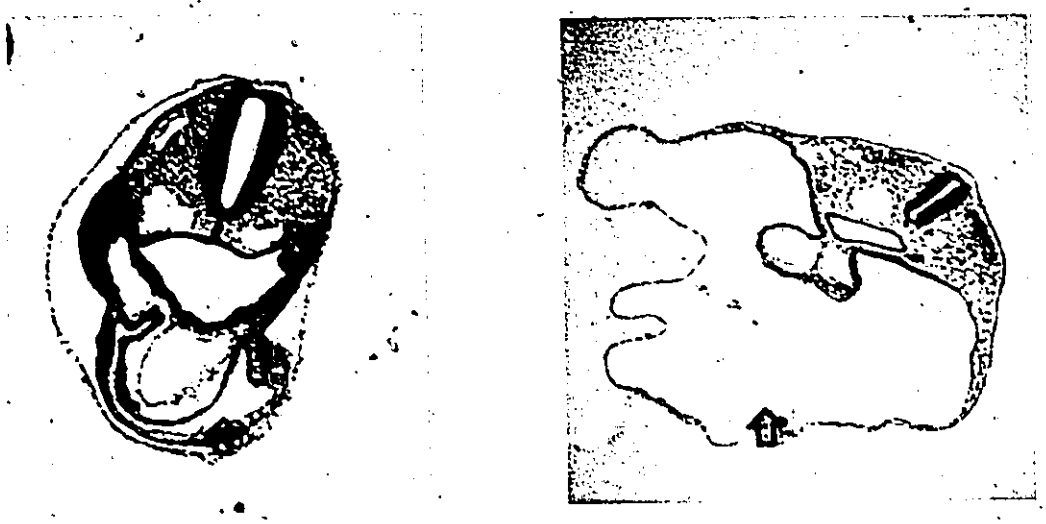
(a) (b)

(a) Control embryo illustrating completely closed neural tube, optic vesicles and notocord in head region (30x).

(b) Embryo from a female fed the 1.0% pCPA test diet with an open neural tube in the head region. This photograph is slightly posterior to the one in (a). This embryo was also judged to be retarded and the smaller size is evident (30x).

Figure 25

(a) (b)



(a) Embryo from a Purina control female demonstrating a normal pericardical cavity (arrow). The amnion is still present in this specimen (30x).

(b) Embryo from a female fed the 3.5% Phe test diet with an abnormally large pericardial cavity (arrow). This embryo was living but judged to be developmentally retarded (30x).

Figure 26

(a)

(b)

(a) A bizzare trilaminar embryo from a female fed the 3.5% Phe diet. No normal embryonic structures were evident and there were no areas of tissue necrosis observed. (100x).

(b) A similar embryo from the same female. The three cell layers may represent the three germ layers (100x).

that retarded embryos may have been present in group (a).

ANOVA of the litter scores for retarded embryos revealed a significant effect for the test diet group ($F = 12.558$, $df = 4, 11$, $p = 0.0004$) and multiple comparisons indicated that the 1.0% pCPA group had significantly more retarded embryos than all other groups.

The number of somites is another indication of developmental progress during early pregnancy. Table 42 indicates that groups (a), (b), and particularly (c), had lower mean somite counts compared to groups (d) and (e). ANOVA of the mean number of somites per litter revealed a significant group effect ($F = 3.911$, $df = 4, 13$, $p = 0.0268$). A Student-Newman-Keuls multiple range test showed that in group (c) the number of somites was significantly less ($p < .05$) than in groups (d) and (e) but not significantly less than in groups (a) and (b). Part of the decrease in the mean number of somites in groups (a) and (c) is a consequence of those implantations in which no distinct embryo could be detected and which therefore were scored as having zero somites.

Maternal Phe and PA: The mean plasma PA and Phe levels as measured fluorometrically and by paper chromatography for the treatment period and the pre-test diet day (day 1) are illustrated in Figure 27. Mean daily PA levels in the 3.5% Phe 1.0% pCPA group, as measured fluorometrically, were greater than all other groups and ranged from 15.5 to

18.2 mg/100 ml. These data are comparable to previous results in both pregnant and nonpregnant animals (Tables 12, 18, 19, 30, 32, 35, 41). Group (c), the 1.0% pCPA group, had moderately elevated mean PA levels, ranging from 10.4 to 12.0 mg/100 ml while group (b), the 3.5% Phe group, had mean fluorometric Phe levels ranging from 2.0 to 6.8 mg/100 ml. Both groups (d), 0.025% Asp, and (e), Purina chow, had similar mean fluorometric Phe levels which did not rise above 2.0 mg/100 ml. Two-way ANOVA, (with repeated measures and covariate analysis), using logarithmic transformation of the fluorometric PA, Phe data, revealed a significant effect only for the test diet group ($F = 31.722$, $df = 4, 12$, $p << 0.001$) and an insignificant covariate indicating that there were not significant group differences prior to the test diet treatments. Two-way ANOVA of the log transformed fluorometric PA, Phe data from the 3 test diet days (without covariate) also indicated a significant effect for the treatment group ($F = 92.224$, $df = 4, 14$, $p << 0.001$) but no effect for the day of blood sampling ($F = 2.576$, $df = 2, 28$, $p = 0.094$). Multiple comparisons of the fluorometric Phe, PA data by the Student-Newman-Keuls procedure showed that groups (a) and (c) did not differ significantly from one another but had significantly greater PA levels during the treatment period than groups (b), (d) and (e). In addition, group (b) had fluorometric Phe levels significantly higher than both groups (d) and (e). One-way ANOVA of the transformed pooled mean

Figure 27. Series E, Experiment 3. Mean daily maternal plasma phenylalanines as measured fluorometrically (upper panel) and by paper chromatography (lower panel). All samples in groups (d) and (e) had phenylalanine concentrations which were undetectable by paper chromatography.

Group (a) 3.5% Phe 1% pCPA
Group (b) 3.5% Phe
Group (c) 1.0% pCPA
Group (d) 0.025% Asp
Group (e) Purina

Figure 28. Series E, Experiment 3. Mean daily plasma maternal tyrosine levels as measured by fluorometry (upper panel) and by paper chromatography (lower panel). All values were below the level of detection in groups (c), (d) and (e). Curves as in Figure 27.

Figure 27

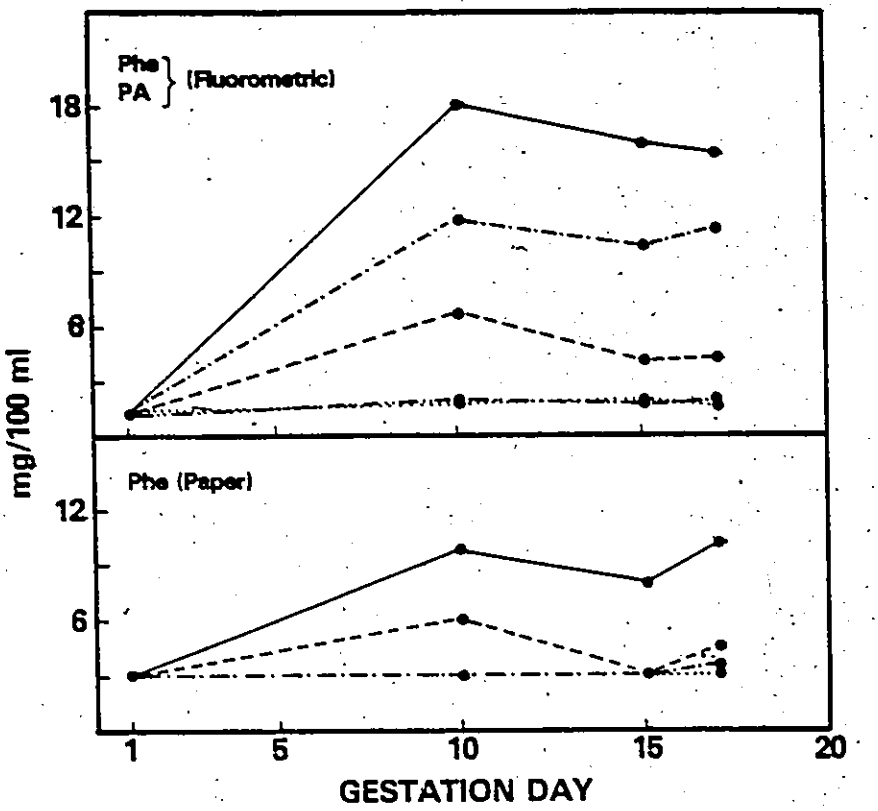
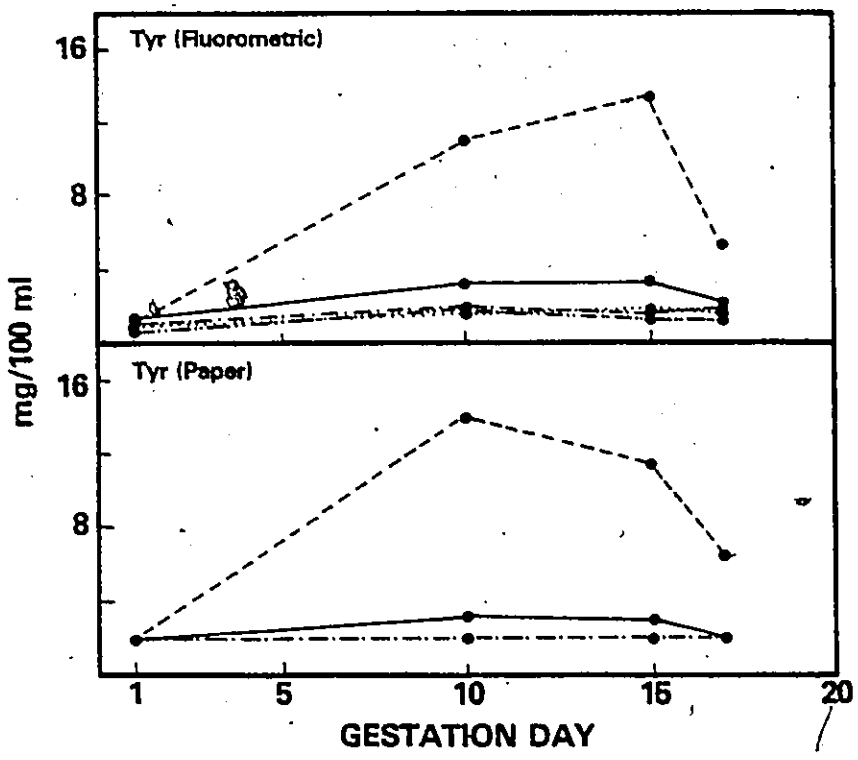


Figure 28



fluorometric PA, Phe levels during the test diet period also revealed a significant effect for the test diet group ($F = 174.682$, df 4, 14, $p << .0001$) and the multiple range comparison indicated that group (a) > (c) > (b) > (d) and (e), all at the 0.05 level. Table 44 illustrates these pooled mean data for each treatment group.

Paper chromatographic estimation of plasma Phe indicated that the 3.5% Phe 1.0% pCPA group also had the highest elevation of Phe, with daily means ranging from 8.0 to 10.2 mg/100 ml (Figure 27). In contrast to the fluorometric data, the 3.5% group, (b), had higher mean Phe levels than did the 1.0% pCPA group, (c), (Figure 27). Both groups (d) and (e) had Phe levels which were not detectable by the paper chromatographic method. The paper values, ie Phe, comprised from 59 to 67% of the mean fluorometric PA levels in the 3.5% Phe 1.0% pCPA group, but only 25 to 31% in the 1.0% pCPA group. ANOVA (covariate with repeated measures) of the logarithm transformed paper chromatographic Phe data revealed only a significant effect for the test diet group ($F = 15.104$, df = 4, 12, $p < 0.001$) as did similar two-way ANOVA (without covariate analysis) of the test diet period data, days 10, 15, and 17, ($F = 18.350$, df = 4, 14 $p < .001$). In neither ANOVA was there a significant effect for the day of blood sampling. On days 10, 15, and 17, Student-Newman-Keuls comparisons indicated that Phe levels in group (a) were significantly ($p < .05$) greater than all

Table 44 Series E, Experiment 3. Mean (±S.D.) maternal plasma phenylalanines and tyrosine for each study group during the test diet period. Solid bars under means indicate those groups not differing at the 0.05 level (Student-Newman-Keuls multiple range).

	Method*	(a) 3.5% Phe 1.0% pCPA	(b) 3.5% Phe	(c) 1.0% pCPA	(d) 0.025% Asp	(e) Purina
Phenylalanine	Fluoro.	16.58±2.55	5.08±1.04	11.20±1.75	1.82±0.80	1.88±0.20
	Paper	9.3±2.5	4.5±0.6	3.2±0.3	3.0±0	3.0±0
Tyrosine	Fluoro.**	2.98±1.14	9.95±13.42	1.87±0.33	1.70±0.11	1.50±0.20
	Paper	2.8±1.5	10.7±15.1	2.0±0	2.0±0	2.0±0

* fluoro = fluorometric assay, paper = paper chromatographic method.

** groups (a), (c), (d) and (e) did not differ significantly from one another.

other groups which in turn did not differ significantly from one another. One-way ANOVA of the pooled mean paper chromatographic Phe levels (transformed) during the test diet period also revealed a significant group effect ($F = 43.041$, $df = 4, 14$, $p < .0001$), and multiple range comparisons further indicated that Phe in group (a) was significantly greater than group (b), which in turn was significantly greater than the remaining groups, (c), (d) and (e) (Table 44).

The mean values for plasma Tyr on each day of blood sampling are illustrated in Figure 28. The only group which had marked elevation of plasma Tyr was the 3.5% Phe group (b), while the 3.5% Phe 1.0% pCPA animals had only slightly increased Tyr. Tyr was virtually unchanged during the course of the study in groups (c), (d) and (e). Two-way ANOVA of the logarithm transformed fluorometric Tyr data revealed a significant covariate ($p = 0.001$) indicating that groups differed significantly prior to treatment. In addition, there was a significant treatment group effect ($F = 5.059$, $df = 4, 12$, $p = 0.013$) in this ANOVA. When the pre-test diet data were excluded and ANOVA performed on the transformed data for days 10, 15 and 17, a significant days effect was found ($F = 3.816$, $df = 2, 28$, $p = 0.034$) but the group effect just failed to reach significance ($F = 3.085$, $df = 4, 14$, $p = 0.051$). Student-Newman-Keuls comparisons showed that when all groups were considered, the fluorometric Tyr was significantly ($p < .05$) greater on day 10 than days 15 and

17, which in turn did not differ significantly. The fluorometric Tyr data were analyzed further by doing one-way ANOVA on the pooled test diet period mean Tyr levels for each group. This ANOVA revealed a significant group effect ($F = 3.585$, $df = 4, 14$, $p = 0.0326$). Multiple comparisons showed that over the entire test diet interval, Tyr in groups (a) and (b) did not differ significantly, but only group (b), 3.5% Phe, was significantly greater than groups (c), (d) and (e) (Table 44). In summary, the analysis of the fluorometric Tyr data suggest that Tyr tended to be higher on day 10, independant of the group, and that the test diet was a significant factor in determining differences in plasma Tyr. Group (b) was the only group to have Tyr levels significantly greater than groups (c), (d) and (e).

Maternal Tyr was also estimated by paper chromatography. The paper and fluorometric methods agreed reasonably well and the group means on each day of blood sampling are illustrated in Figure 28. Two-way ANOVA of the log transformed chromatographic data revealed a marginally significant effect for the day of treatment ($F = 3.391$, $df = 4, 12$, $p = 0.049$) and an insignificant covariate. When the day 1 data were excluded, a marginally significant interaction between the treatment groups and the day of blood sampling was detected ($F = 2.312$, $df = 8, 28$, $p = 0.048$). Because of this interaction, the data had to be reanalyzed by ANOVA after being broken down by days (across groups) and by groups (across

days). Within each diet group, ANOVA did not reveal any significant differences among the three days. ANOVA of the data on each of the three days of blood sampling revealed a significant group effect only on day 10 ($F = 3.398$, $df = 4, 14$; $p < .05$). Within day 10, the 3.5% Phe group (b) had significantly greater Tyr levels ($p < .05$) than all other groups which in turn did not differ significantly from one another. These data are consistent with the fluorometric Tyr data in suggesting that group (b) had significantly higher Tyr levels and that the day of blood sampling was a significant factor in determining Tyr levels. Gestation day 10 appeared to stand out both with respect to differences between groups and in comparison to days 15 and 17.

On gestation day 17 plasma from a number of females was also analyzed by column chromatography. Table 45 includes the PA, Phe, pCPA, and Tyr levels as measured by fluorometry, paper chromatography and the amino acid analyzer. The two samples from animals in the 3.5% Phe 1.0% pCPA group had the highest Phe (or PA) concentration by all three methods, although the analyzer values were less than one half of the paper Phe values. pCPA was undetectable in one of these samples and was only 2.89 mg/100 ml in the other. In contrast, the analyzer samples from group (c), 1.0% pCPA test diet, averaged 7.90 mg/100 ml of pCPA but only 2.74 mg/100 ml Phe. In this group the paper chromatographic data were also consistent with the conclusion that approximately

Table 45 Series E, Experiment 3. Maternal amino acids on gestation day 17 as measured by fluorometry, paper chromatography and column chromatography, mg per 100 ml.

Group	Number of Samples	Amino Acid	Fluorometric Datum	Fluorometric Mean	Paper Chromatography* Datum	Paper Chromatography* Mean	Analyzer Datum	Analyzer Mean
(a) 3.5% Phe 1.0% pCPA	2	PA/Phe	14.5 18.6	16.55	10 14	12.0	5.19 4.63	4.910
		Tyr	2.0 2.2	2.10	2 2	2.0	1.56 1.62	1.590
		pCPA					2.89 0	1.445
(b) 3.5% Phe	3	Phe	6.0 5.4 1.5	4.30	6 6 3	5.0	3.42 4.54 2.40	3.453
		Tyr	4.0 13.1 1.6	6.23	2 20 2	6.0	2.69 17.17 0.92	6.927
(c) 1.0% pCPA	3	PA/Phe	9.8 11.6 14.8	12.07	3 3 5	3.7	1.22 2.15 4.85	2.740
		Tyr	1.4 2.5 2.0	1.97	2 2 2	2.0	0.83 2.41 1.81	1.683
		pCPA					7.20 7.27 9.24	7.903

Continued...

Table 45 Series E, Experiment 3
(Con't.)

Group	Number of Samples	Amino Acid	Fluorometric		Paper Chromatography*		Analyzer	
			Datum	Mean	Datum	Mean	Datum	Mean
(d) 0.025% Asp	2	Phe	1.4 2.0	1.70	3 3	3.0	0.55 1.87	1.210
		Tyr	1.6 1.6	1.60	2 2	2.0	0.64 0.91	0.775
(e) Purina	2	Phe	1.5 2.0	1.75	3 3	3.0	1.42 1.88	1.650
		Tyr	1.2 1.4	1.30	2 2	2.0	0.60 0.96	0.780

* Levels below the limit of detection taken as 3 mg% and 2 mg% for Phe and Tyr, respectively.

75% of the fluorometric PA levels were composed of pCPA. In group (a), the fraction of the PA levels made up by pCPA is clearly less than in the 1.0% pCPA group and is probably about 50%, or less. Among the three groups consuming diets without pCPA, the Phe values were comparable by the three methods. Plasma Tyr, as estimated by the three methods, also was similar within each group.

Urine $FeCl_3$ Tests: When the animals voided while being handled, fresh urine was collected and tested for phenylketones with $FeCl_3$. The results of all $FeCl_3$ tests are summarized in Table 46. All urine samples from animals consuming pCPA diets were positive whereas none of the samples from other groups were positive. There was only a single urine sample available from group (b), so it was not possible to adequately assess the relationships between moderate hyperphe in the absence of pCPA treatment and urinary phenylketone excretion.

Yolk Sac Fluid: It was possible to collect small amounts of yolk sac fluid from most implantation sites. Depending on the volume of the sample, it was analyzed for PA, Phe and Tyr by the fluorometric and/or the paper chromatographic methods. Fluid was analyzed by at least one method in all but 2 litters, one of which had aborted. As was done with other data from individual embryos, a mean was calculated for each litter and used for analysis. Figure 29 summarizes the mean yolk sac fluid amino acid levels in each group as

Table 46 Series E, Experiment 3. Urine $FeCl_3$ tests. Frequency of positive tests in each test diet group.

Group	Number Females Tested	Number positive/number samples
(a) 3.5% Phe 1.0% pCPA	3	11/11
(b) 3.5% Phe	1	0/1
(c) 1.0% pCPA	3	3/3
(d) 0.025% Asp	2	0/2
(e) Purina	1	0/1

measured by both methods, as well as the corresponding maternal day 17 plasma levels. The figure suggests that yolk sac Phe levels are elevated above the normal range in group (a) and possibly (b), while Tyr levels were dramatically increased in group (b) and only moderately in groups (a) and (c). These possibilities were examined by subjecting the yolk sac fluid amino acid data (with log transformation) to ANOVA and Student-Newman-Keuls multiple comparisons.

ANOVA of the fluorometric PA/Phe data indicated a significant effect for the test diet group ($F = 12.396$, df 4, 11; $p = .0005$) and the multiple comparison showed that group (a) had significantly ($p < .05$) higher PA levels than all other groups. In addition, group (e) was found to have Phe levels significantly ($p < .05$) lower than all other groups. ANOVA of the paper Phe levels also revealed a highly significant group effect ($F = 42.368$, df = 4, 12, $p < .0001$). The multiple comparisons again indicated that group (a) had significantly greater Phe levels than all other groups ($p < .05$) and, in addition that group (b) had significantly higher Phe levels than the remaining groups. These data indicate that the 3.5% Phe 1.0% pCPA group had significantly greater PA and Phe in embryonic fluid than did all other groups. In addition, it appears that yolk sac fluid concentrates Phe relative to maternal plasma as Phe was greater in the yolk sac fluid in groups (a), (b), (d) and (e). This relationship was true in all the litters

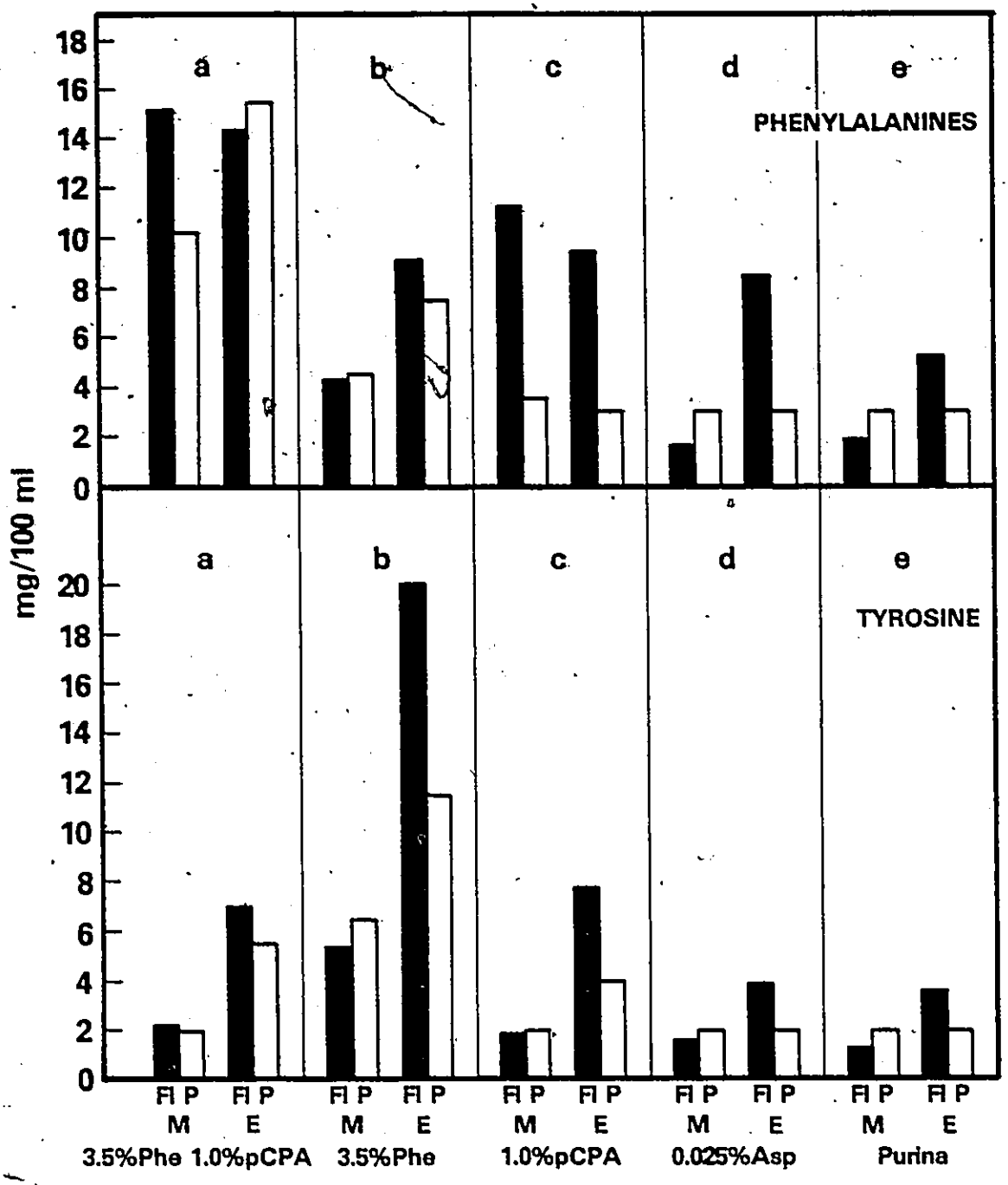


Figure 29. Series E, Experiment 3. Gestation day 17 mean maternal plasma (M) and yolk sac fluid (E) phenylalanines and tyrosine of each group as measured fluorometrically (Fl) and by paper chromatography (P).

in these groups; and in only one litter in group (c), was the Phe greater in plasma than yolk sac fluid (5 mg/100 ml vs 3 mg/100 ml paper chromatographic value).

Tyr was uniformly greater in yolk sac fluid than in maternal plasma in all litters, whether estimated fluorometrically or chromatographically. When the transformed fluorometric Tyr data were subjected to ANOVA, a significant effect for the diet group was found ($F = 6.473$, $df = 4, 11$, $p = .0063$). Multiple comparisons of these data indicated that group (b), 3.5% Phe diet, had significantly greater Tyr levels ($p < .05$) than groups (d) and (e). No other groups differed significantly from one another. Similar ANOVA of the paper chromatographic Tyr data however, did not reveal a significant effect for the diet group ($F = 1.555$, $df = 4, 12$, $p = .249$).

The degree of association between gestation day 17 maternal plasma amino acids and yolk sac fluid amino acids as measured by similar methods was estimated by use of a 2 cell by 2 cell frequency table. For each assay method the mean yolk sac concentration and the corresponding maternal plasma concentration were assigned to one of the four cells on the basis of whether the concentration exceeded or fell below a cut off point criterion value (see sample table on bottom of Table 47). The degree of association between the maternal and yolk sac levels was then assessed by using the frequencies in each cell to compute the significance using

Fisher's Exact test. This approach indicated that the maternal plasma and yolk sac fluid PA and Phe were significantly associated as were the Tyr levels as measured by fluorometry (Table 47). Paper chromatographic estimation of Tyr in plasma and yolk sac fluid was not significantly associated. These data suggest that as maternal Phe and Tyr increase, yolk sac fluid Phe and Tyr also tend to increase. The data were too few to permit valid regression or correlation analyses.

Maternal Body Weight: The mean daily body weight for each group is illustrated in Figure 30. The figure suggests that although all groups gained weight during the study interval, groups (a) and (c) did so less rapidly. It also appears that in spite of the random allocation of animals into diet groups, the groups had different mean weights on gestation day 1. The data were subjected to ANOVA with repeated measures following logarithmic transformation to stabilize the variance. This ANOVA revealed a highly significant interaction between gestation day and the test diet group ($F = 2.296$, $df = 64, 224$, $p < .0001$). The rates of weight gain (slope) in groups (a) and (c) were similar to one another but different from the rates of groups (b), (d), and (e), which in turn, appear to be approximately parallel to each other (Figure 30). Separate two-way ANOVA was therefore performed on the transformed data from groups (a) and (c) and on the data from groups (b), (d) and (e). Using

Table 47 Series E, Experiment 3, Association between maternal plasma (gestation day 17) and yolk sac fluid amino acids. Probability refers to that calculated using Fisher's Exact test in a 2 x 2 table. Each amino acid value was allocated to a cell in the 2 x 2 table by whether it fell above or below the criteria values. See sample 2 x 2 table below.

	Method	CUT OFF CRITERIA (mg/100 ml) Maternal Plasma	Yolk Sac Fluid	Probability*
Phenylalanines	Fluorometric	≤10, >10	≤10, >10	p = 0.019
	Paper Chromatographic	≤3, >3	≤3, >3	p = 0.017
Tyrosine	Fluorometric	≤2, >2	≤5, >5	p = 0.028
	Paper Chromatographic	≤4, >4	≤4, >4	N.S.

* N.S. = not significant

Sample 2 x 2 Table: Phenylalanines fluorometric assay

		Maternal plasma mg/100 ml ≤10	>10	sum	
Yolk Sac Fluid	≤10	9	0	9	
	>10	3	4	7	p = 0.019
	sum:	12	4	16	samples total

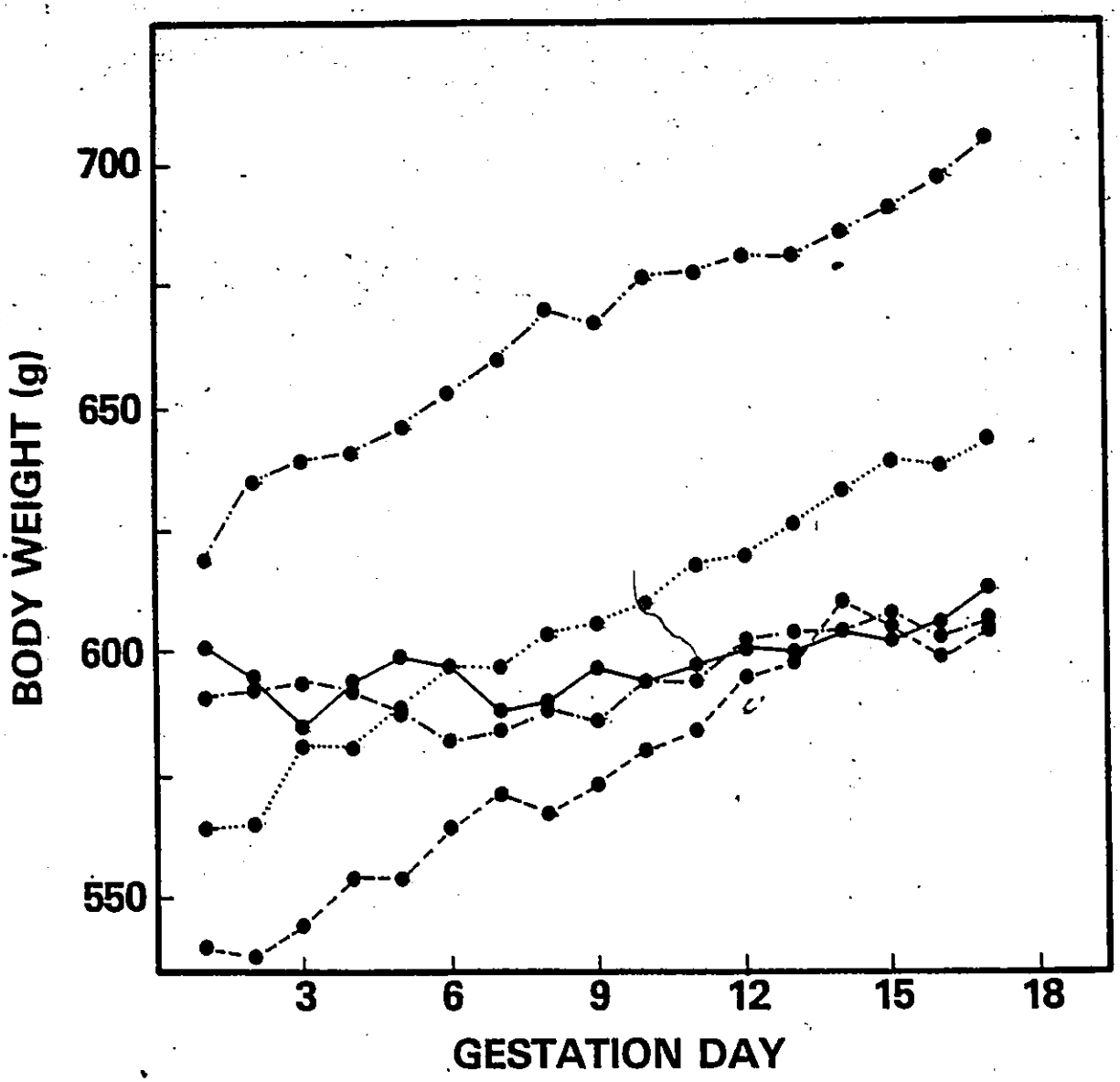


Figure 30. Series E, Experiment 3. Mean daily maternal body weight of each group.

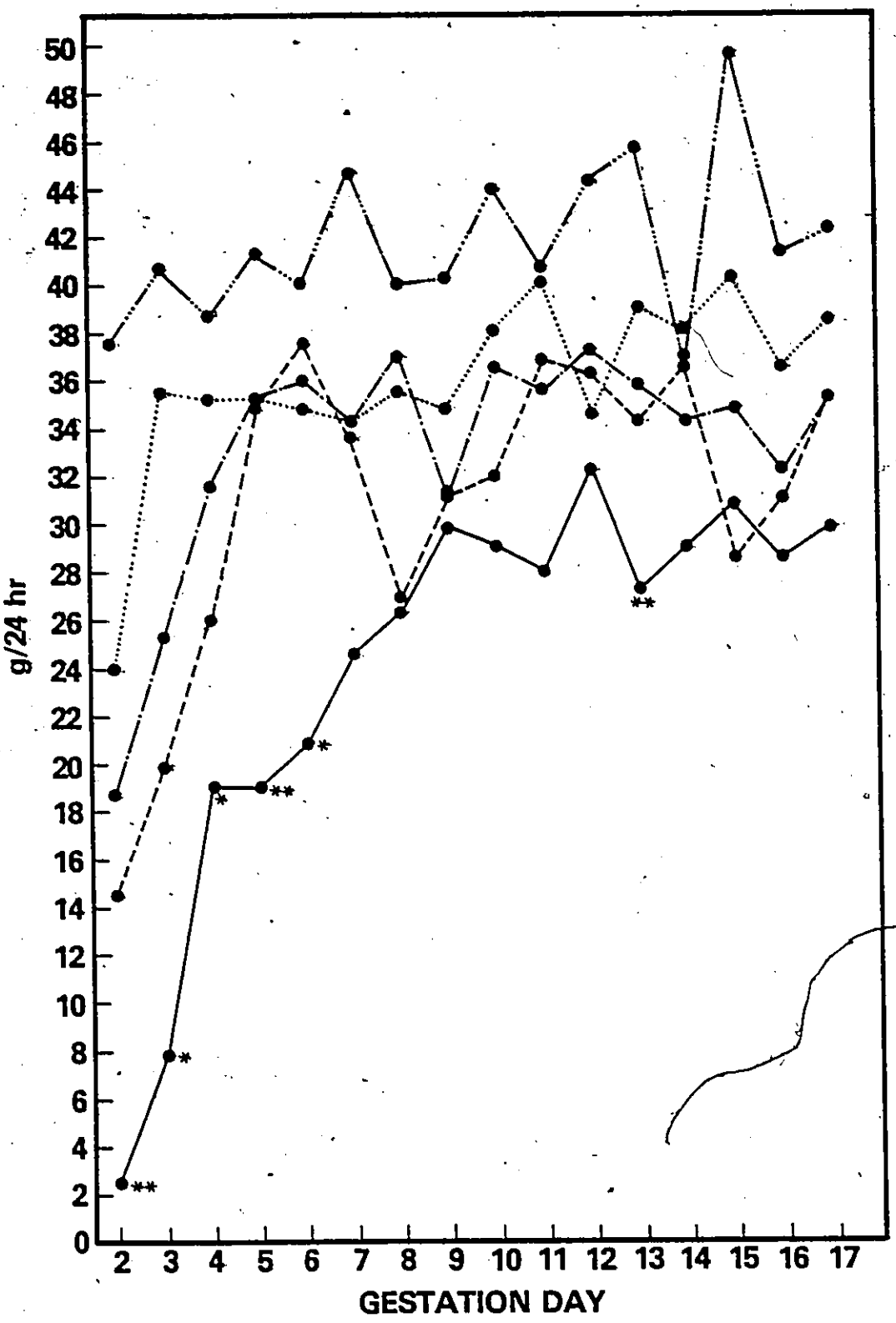
Group (a) ——
Group (b) – – –
Group (c) —·—
Group (d) .....
Group (e) —··—

the day 1 body weight as the covariate ($p = .018$), ANOVA of the data from groups (a) and (c) revealed a significant effect for gestation day ($F = 2.654$, $df = 15, 90$, $p = .002$) but no effect for the diet group ($F = .077$, df 1, 5). Similar ANOVA on the data from groups (b), (d) and (e) also revealed a significant covariate ($p = .029$), a significant days effect ($F = 25.561$, $df = 15, 120$, $p < .0001$), but no effect for the diet group ($F = .544$, $df = 2, 4$). The significant covariates indicate that the groups did indeed begin pregnancy with significantly different body weights. These analyses also indicate that for all groups the weight changed significantly during the 17 day test diet period. In addition, the two groups consuming diets containing pCPA, (a) and (c), did not differ from one another but were clearly different from the remaining groups, (b), (d) and (e).

Daily Food Intake: The mean daily food intake of each group is illustrated in Figure 31. Although there was considerable day to day variation in the mean food intake, it is apparant that the groups fed Phe and/or pCPA test diets tended to eat smaller amounts when first presented with the test diets. The data were transformed to logarithms to stablize the variance and subjected to two-way ANOVA which indicated a significant interaction between the gestation day and the test diet group ($F = 1.408$, $df = 60, 218$, $p = .040$). In order to permit analysis of the effects of the test diets independantly from the effects of the number of

Figure 31. Series E, Experiment 3. Mean daily maternal food intake of each group. *Group (a) significantly less than Groups (c), (d), (e), $p < .05$. **Group (a) significantly less than Groups (b), (c), (d), and (e), $p < .05$. Curves as in Figure 30.

Figure 31.


g/24 hr
GESTATION DAY
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2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
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days on the diets, one-way ANOVAs were performed across all days for each diet group and across all diet groups for each day. It was found that within each test diet group there were significant effects for the day of gestation only in groups (a) ($F = 8.526$, $df = 15, 43$, $p = .001$), (c) ($F = 5.864$, $df = 15, 44$, $p = .001$), and (d) ($F = 2.286$, $df = 15, 43$, $p = .017$). When the food intake of the diet groups was compared by ANOVA on each of the 17 gestation days, significant effects for the diet group were found on days 2 through 6 and again on day 13. Student-Newman-Keuls multiple range tests were used on each of these days to determine which groups differed significantly ($p < .05$). On these days group (a), the 3.5% Phe 1.0% pCPA diet group, had a significantly lower food intake than either groups (b), (c), (d) and (e) or groups (c), (d) and (e) (see Figure 31). No other diet groups were found to differ significantly. When the transformed data from all groups for days 7-17 was subjected to ANOVA no significant effects for days or diet group were found, nor was there a significant interaction of days with diet group.

In summary, the number of days on the test diets was a significant determinant of the daily food intake and on days 2 through 6 and 13, group (a) had a significantly lower dietary intake than other groups. After day 7, when all groups were considered together, neither the composition of the diet nor the gestation day were significant factors in daily food intake.

Maternal Liver Weight: Maternal livers were weighed immediately following removal from the animal and the mean weight for each group is shown in Table 48. One-way ANOVA of these data did not reveal any significant effect for the treatment group ($F = 0.880$, $df = 4, 13$).

Hepatic Ascorbic Acid: The mean ascorbic acid content of the maternal liver tissues is also shown in Table 48. These data were subjected to one-way ANOVA and no significant effect for the diet group was detected ($F = 0.292$, $df = 4, 14$). These data show that the daily ascorbic acid supplement given to animals in groups (a) through (d) was sufficient to prevent ascorbic acid deficiency.

Hepatic Phe Hydroxylase Activity: The mean Phe hydroxylase activity in the presence of the synthetic cofactor DMPTH is shown for each group in Table 48. The two groups consuming diets containing pCPA had enzyme activities between 32 and 35% of the Purina control tissue. These data were subjected to ANOVA and a significant effect for the diet group was found ($F = 17.396$, $df = 4, 18$, $p < .0001$). A Student-Newman-Keuls multiple range test indicated that the activity of groups (a) and (c) did not differ significantly from one another, but that both groups had significantly less activity than groups (b), (d) and (e) ($p < .05$). The latter groups, none of which received any pCPA, did not differ significantly from each other. These data indicate that pCPA inhibits Phe hydroxylase

Table 48 Series E, Experiment 3. Mean (±S.D.) maternal liver weight, hepatic ascorbic acid and hepatic phenylalanine hydroxylase activity in each group.

Group	Number of Females	Liver Weight (g)	Hepatic Ascorbic Acid mg/100 g/wet weight	Hepatic Phenylalanine Hydroxylase* μMole Tyr/hr/g wet weight
(a) 3.5% Phe 1.0% pCPA	4	23.0±2.4	28.75±9.00	8.63±3.75 (34.5%)
(b) 3.5% Phe	4	23.6±4.4	29.78±7.38	20.57±7.77*** (82.2%)
(c) 1.0% pCPA	4	27.6±4.8**	28.55±4.88	7.98±3.02 (31.9%)
(d) 0.025% Asp	4	25.5±1.8	28.82±5.73	25.12±1.82 (100.4%)
(e) Purina	3	27.1±6.4	33.30±4.01	25.03±1.64 (100%)

* (% Purina control)
** only 3 livers were weighed in group (c)
*** only 3 livers in group (b) assayed for Phe hydroxylase

to a similar extent in both pregnant and nonpregnant guinea pigs (cf Tables 11, 34).

Association Between Embryo Status and Amino Acids and Food Intake: The degree of association between the frequency of live, abnormal, or retarded embryos with maternal amino acids, yolk sac amino acids, and maternal food intake was assessed using Fisher's Exact test as described above for yolk sac amino acids. For these analyses a litter was classified on the basis of the litter score for live, abnormal, and retarded embryos (see Table 42), with the criterion for the 2 by 2 tables being 2.0 versus less than 2.0. (A score of 2.0 indicates that all embryos in that litter were alive, normal, or not retarded). Litter scores were paired with the appropriate amino acid level and assigned to a cell in the 2 by 2 table on the basis of the cut-off criteria for that amino acid. The embryonic data were tested with yolk sac PA, Phe, and Tyr, gestation day 17 maternal PA, Phe and Tyr, and the mean of days 10, 15 and 17 maternal plasma PA, Phe and Tyr, all as measured by both fluorometry and paper chromatography. In addition, the three embryonic parameters were tested for association with the mean daily food intake of the corresponding female during 2 intervals, gestation days 2 through 6, and days 7 through 17. The cut off criterion for food intake was 30 g/24 hours. Table 49 shows all the Fisher's tests which indicated a significant association

between the embryonic variables and the various amino acid data as well as the cut-off criteria used in each of these significant tests. As indicated in the table, only maternal PA and Phe was significantly associated with either dead or abnormal litters. No tests involving Tyr data or daily food intake showed significant associations nor did any tests involving the frequency of retarded litters (all p values > 0.05). There appears to be a relationship between maternal plasma Phe and embryonic mortality and abnormality, but no relationship between maternal food intake and defective embryos could be detected. The significant association between maternal Phe concentration and abnormal embryos is particularly noteworthy in view of the high frequency of abnormal litters in the 3.5% Phe group. These data suggest that mild hyperphe, even in the absence of pCPA treatment, may be related to the development of malformed embryos.

Table 49 Series E, Experiment 3. Significant associations between Live or Abnormal embryos and maternal plasma phenylalanines, using Fisher's Exact test in a 2 x 2 table. There were no significant associations between maternal plasma Tyr or food intake and the embryonic variables.

Embryonic Variable	Amino Acid Parameter: Amino Acid, Method, Source, Day. Cut-off Criteria.	Probability
Live/Dead	PA/Phe, Fluorometric, Maternal Day 17. ≤8, >8	p = 0.0048
	PA/Phe, Fluorometric, Maternal Mean Days 10, 15, 17. ≤8, >8	p = 0.0048
Normal/ Abnormal	PA/Phe, Fluorometric, Maternal, Day 17. ≤8, >8	p = 0.035
	PA/Phe, Fluorometric, Maternal Mean days 10, 15, 17. ≤8, >8	p = 0.035
	Phe, Paper, Maternal Mean days 10, 15, 17. ≤3, >3	p = 0.014

Discussion

A number of criteria for experimental models of maternal PKU have already been outlined and these will be considered in discussing the present study. The production of stable maternal hyperphe is a basic requirement of any model and the elevation of Phe should approach 15 mg/100 ml, the level associated with substantial risk to the human fetus (Table 6). Since the fetal to maternal ratio of plasma Phe in the guinea pig in contrast to the data of Christensen and Streicher (1948), has been shown to be similar to that of humans, (Ghadimi and Pecora 1964; Cockburn et al 1970; Matsuda et al 1977) plasma Phe in pregnant guinea pigs must also be near 15 mg/100 ml to reproduce the fetal Phe levels found in man (Bovier-Lapierre et al 1974; Bush and Dukes 1975; Cockburn et al 1972; Huntley and Stevenson 1969; Thomas et al 1971). The fetal maternal ratios of Phe and Tyr observed in the guinea pigs in the present study were also similar to those reported in the rat and monkey (Lines and Waisman 1971; Wapnir and Dierks-Ventling 1971; Kerr and Waisman 1966; Kerr et al 1968; Kerr 1968) and probably differ from those in the guinea pig reported by Christensen and Streicher because these workers estimated α-amino nitrogen rather than Phe and Tyr individually. It was for

this nonspecific measure of amino acids that Christensen and Streicher observed a 5:1 fetal to maternal ratio. In a more recent study Young (1971) has reported that both Phe and Tyr in fetal guinea pig plasma are approximately twice the level found in maternal plasma. Young also measured other amino acids in fetal and maternal plasma, and all but cystine were higher in fetal blood. Young did not report the gestational age of the guinea pigs used in her study. The present work however, has clearly shown that the fetal to maternal ratios for both Phe and Tyr in the guinea pig do not approach 5:1 at any time during the last trimester of pregnancy.

Initial efforts to induce hyperphe in guinea pigs involved injection of Phe and the Phe hydroxylase inhibitor pCPA (Lipton et al 1967 ; Koe and Weisman 1966). This approach has been used by a number of other investigators in efforts to produce hyperphe in both pregnant (Foote and Tao 1968; Copenhaver et al 1970; 1973; 1974; 1978; Schalock and Copenhaver 1973; Glazer and Weber 1971a; Andersen 1976) and non pregnant rats (eg: Andersen and Avins 1976; Delvalle et al 1978; Gunter et al 1972; Schalock et al 1975; Andersen et al 1974; Prohaska and Wells 1974; Butcher et al 1977; Loo and Scotto 1977). In most of the studies with pregnant rats maternal blood PA levels (Phe was not separated from pCPA) were found to be in the range associated with significant risk to the human fetus (Copenhaver et al 1973;

1974; Schalock and Copenhaver 1973; Glazer and Weber 1971a; Andersen 1976). The blood samples in which these high PA levels were found however, were all collected within 4 to 8 hours of Phe injection. In those instances when blood was obtained at longer intervals following the Phe injection, plasma PA levels (of which a significant fraction may in fact be pCPA and not Phe) were uniformly less than 8 mg/100 ml (Copenhaver et al 1974; Schalock and Copenhaver 1973; Andersen 1976). The present study has clearly demonstrated that adult female guinea pigs do not maintain elevated Phe levels for longer than 10 hours when injected with large doses of both Phe and pCPA (Series B, Appendix A). These guinea pigs received up to 750 mg/kg of pCPA during the 24 hours preceeding Phe injection, a dosage substantially larger (in mg/kg) than has been required in rat studies. In the rat, a single injection of 360 mg/kg of pCPA has been shown to maximally inhibit hepatic Phe hydroxylase (Delvalle and Greengard 1976). In addition, the enzyme activity has been shown to remain maximally inhibited for at least 48 hours following the injection of pCPA (Guroff 1969; Delvalle and Greengard 1976; Koe and Weissman 1966; Gal and Millard 1971). Experiments 2 through 8 of Series B however, provide indirect evidence that in pCPA-injected guinea pigs Phe hydroxylase probably remains maximally inhibited for less than 12 hours (Figures 1 to 4). The shorter duration of pCPA-induced Phe hydroxylase inhibition in guinea pigs is suggested by the

progressively higher blood Phe levels observed as the frequency of the pCPA injections was increased. The possibility that rapid recovery of Phe hydroxylase activity occurs in pCPA treated guinea pigs is not inconsistent with the enzyme activity detected in the guinea pigs given similar dosages of pCPA via supplemented test diets (Series C, Experiments 1 and 5, Series E, Experiment 3). In these studies Phe hydroxylase activity was found to be reduced to about 30% of normal in guinea pigs fed 1.0% pCPA diets. Since guinea pigs have been shown to eat at least every 90 minutes (Hirsch 1973), the dietary administration of pCPA can be considered approximately equivalant to administering pCPA at least every 90 minutes. The magnitude of Phe hydroxylase inhibition observed in these guinea pigs is comparable to that found in rats fed pCPA supplemented diets, however, diets containing only 0.12% pCPA are required to similarly inhibit Phe hydroxylase in rats (Berry et al 1975). It is clear that to maximally inhibit hepatic Phe hydroxylase, guinea pigs, compared to rats, require not only more pCPA (about 650-800 mg/kg/day, Series C, Experiment 1), but also more frequent administration of pCPA.

The mechanism of this apparant species difference in the response to pCPA is not clear although a number of possible explanations can be considered. It is unlikely however, that poor absorption of pCPA by guinea pigs is a major factor since the difference was evident even when IP

administration of pCPA was used (Series B and Appendix A). It may be that the guinea pig rapidly excretes pCPA and/or its metabolites. Strongly positive, but rapidly fading, urine $FeCl_3$ tests were consistently observed in guinea pigs given pCPA even in the absence of hyperphe (Series B, Series C, Series E, Appendix A). Guinea pigs excreted $FeCl_3$-positive urine within 6 hours of pCPA administration (Series B). The identity of the $FeCl_3$-positive substance(s) is not known with certainty, however it may be p-chlorophenylpyruvic acid. When pCPA is added directly to guinea pig urine it does not react positively with $FeCl_3$ and p-hydroxyphenylpyruvic acid and homogentisic acid, both of which may produce a transiently positive $FeCl_3$ test (Hsia and Inouye 1966), are unlikely to be present in urine except when plasma Tyr is elevated. In Series B, positive $FeCl_3$ tests were repeatedly found following pCPA treatment in the presence of normal plasma Tyr levels. Rats have been shown to convert pCPA to p-chlorophenylpyruvic acid and guinea pigs have also been reported to transaminate pCPA (Koe 1971; Gal et al 1970; Flatlow 1910 cited by Tong et al 1973). In addition, rat liver has been shown to transaminate pCPA in vitro (Fuller et al 1972; Tong et al 1973) and p-chlorophenylpyruvic acid has been recovered from human urine following pCPA administration (Koe 1971). The rapid elimination of pCPA as p-chlorophenylpyruvic acid, and possibly other pCPA catabolites, remains an attractive but as yet unproved explanation of

the guinea pig's diminished enzyme response following pCPA treatment.

It is also possible that the mechanism of enzyme inhibition by pCPA differs in the rat and the guinea pig. pCPA is known to inhibit Phe hydroxylase irreversibly in the rat and new protein synthesis, presumably of Phe hydroxylase, appears to be required to reverse the pCPA-induced enzyme inhibition (Guroff 1969; Gal and Millard 1971; Miller et al 1976). There are no data to indicate whether the mechanism of Phe hydroxylase inhibition in the guinea pig differs from that in the rat, and until such data become available this possibility can not be further evaluated.

In spite of these differences in the response of rats and guinea pigs to pCPA, stable hyperphe in the range associated with a high risk to the human fetus was produced in guinea pigs by dietary administration of both pCPA and Phe (Series C, Experiments 1, 2, 4, 5, Series E, Experiments 2 and 3). Plasma PA levels were consistently elevated above 20 mg/100 ml and separate estimation of Phe showed that Phe was often above 15 mg/100 ml (Figures 5, 8, 8.1, 9, 10, Table 12, 13, 18, 19, 26, 32). It was possible to produce hyperphe with only moderate elevations of plasma Tyr. Significant elevations of maternal plasma Tyr is a factor which has confounded studies which have not attempted to inhibit Phe hydroxylase (eg: Auerbach et al 1958; Boggs and Waisman 1962, 1964; Iijima et al 1975; Wapnir et al 1977). The importance of Phe hydroxylase inhibition in the prevention

of hypertyrosinemia was clearly demonstrated by the high Tyr levels found in animals fed Phe but without concomitant pCPA administration (Series C, Experiment 1, Series E, Experiment 3). Even though Phe hydroxylase activity was reduced to about 30% of normal, the remaining functional enzyme was still able to convert significant amounts of the dietary Phe load to Tyr. This activity was illustrated by the clear trend toward higher plasma Tyr levels with increasing dietary Phe supplementation (Series C, Experiment 2). In the present study the optimum dietary Phe supplement was determined, in part, on the basis of minimizing Tyr elevation. In addition, plasma Tyr levels were minimized by selecting the dietary pCPA supplement which maximally inhibited hepatic Phe hydroxylase. It is of note that some workers have treated pregnant rats with doses of pCPA well below those shown by Delvalle and Greengard (1976) to maximally inhibit rat liver Phe hydroxylase (Schalock and Copenhaver 1973; Copenhaver et al 1978; Andersen 1976). These workers did not assay Phe hydroxylase activity in either pregnant or similarly treated nonpregnant animals and it is likely these animals retained substantial Phe hydroxylase activity. Even with the methods used in the present study, plasma Tyr averaged 4 to 5 mg/100 ml in nonpregnant and 3 to 4 mg/100 ml in pregnant animals fed the optimum test diet. Although these Tyr levels are higher than those found in women with PKU, they are lower than those reported in

experimental studies of maternal PKU in which adequate maternal hyperphe was produced but without the use of pCPA (Boggs and Waisman 1964; Iijima et al 1975; Wapnir et al 1977; Kerr and Waisman 1966; Waisman 1968; Kerr et al 1968; Chamove and Davenport 1970; Chamove et al 1973). In studies in which pregnant rats were treated with both Phe and pCPA, plasma Tyr levels have been found to be less than 3 mg/100 ml (Berry et al 1975, 1977; Copenhaver et al 1973; 1974). As has already been noted however, a number of other authors did not report maternal Tyr levels and it is therefore not known if the animals used in these studies had significant elevations of Tyr (Foote and Tao 1968; Copenhaver et al 1970, 1978; Schalock and Copenhaver 1973; Glazer and Weber 1971a; Andersen 1976).

In PKU fasting blood Phe levels are always elevated and although fluctuations in Phe concentration occur following Phe ingestion, Phe does not fall below 15 to 20 mg/100 ml (Scriver and Rosenberg 1973). In the present study plasma Phe has been shown to remain elevated for at least 38 days in pregnant guinea pigs and for over 30 days in nonpregnant animals fed the 3.5% Phe 1.0% pCPA test diet (Series E, Experiment 2, Series C, Experiment 2). Not only has hyperphe been shown to persist from day to day but it has also been shown to persist for at least 12 hours within a single day (Series C, Experiment 4). The plasma Phe level in fact, was found to be lowest in the morning, the time when blood

was routinely collected in most experiments, and to rise to levels exceeding 23 mg/100 ml during the subsequent 12 hours (Figure 10). This rise in plasma Phe was not accompanied by a concomitant rise in plasma Tyr (Figures 9, 10). This persistent hyperphe contrasts sharply with the transient hyperphe following injection of Phe and pCPA observed both in the present study (Series B) and in some published studies of experimental maternal hyperphe (Copenhaver et al 1973, 1974; Schalock and Copenhaver 1973; Andersen 1976; Wapnir and Dierks-Ventling 1971). The relevance of these latter studies, and undoubtably also those in which the temporal changes in maternal Phe were not reported (Foote and Tao 1968; Copenhaver et al 1970, 1978), is limited by the wide fluctuations of maternal Phe levels. In most cases Phe returned to near-normal levels prior to the next scheduled treatment, a finding which would not be observed in PKU patients. In addition to the present work, only one other group has induced maternal hyperphe without hypertyrosinemia by dietary administration of Phe and pCPA (Butcher 1970; Berry et al 1975, 1977). These workers did not demonstrate that Phe levels remain elevated throughout a single day, although based on the present data and the eating behavior of rats (Collier et al 1972), there is no reason to suspect that Phe fell to normal levels in these rats. Although Phe was not determined during the early morning hours in the present work, it is doubtful that plasma Phe levels fell significantly

below those observed at 0900 hours, especially when it is recalled that the guinea pig has been found to eat regularly both at night and during the day (Hirsch 1973).

In addition to stable maternal hyperphe, experimental models of PKU should also include information on the urinary excretion of Phe metabolites by pregnant hyperphe animals. In the present work, treated guinea pigs had consistently positive urine $FeCl_3$ tests indicating significant excretion of some phenylketone. It has already been suggested that at least some of this phenylketone may be p-chlorophenylpyruvic acid, a probable pCPA metabolite, which of course is not found in PKU. As Table 9 indicates however, some animals with negative $FeCl_3$ tests prior to Phe administration subsequently were found to have positive tests within 6 hours of Phe injection. These data suggest that significant PPA excretion might also occur in some of the chronically hyperphe guinea pigs fed the 3.5% Phe 1.0% pCPA test diet. The green colour change indicating a positive $FeCl_3$ test usually persisted for only a few minutes in the urine of these guinea pigs, unlike the more stable colour change found in the urine of untreated PKU patients. This observation could mean either that the PPA-$FeCl_3$ reaction product is less stable in guinea pig urine or that PPA was not the $FeCl_3$ positive material. Further study of the urine of hyperphe guinea pigs would be of value both to clarify the identity of the $FeCl_3$ positive material(s) and to more accurately document

the Phe and/or Tyr metabolites excreted by these animals. Relatively few studies of experimental maternal hyperphe have determined whether the pregnant animals excreted metabolites of Phe. In some of these experiments D, L-Phe administration was used and since the D-isomer of Phe can be converted to PPA by D-amino acid oxidase (Scriver and Rosenberg 1973; Karrer and Cahilly 1965) the presence of PPA cannot be used as evidence that significant amounts of Phe have been metabolized by pathways other than via hydroxylation (Auerbach et al 1958; Thompson and Kano 1965). No other experimental studies in which pregnant animals were treated with both Phe and pCPA examined urine for Phe or its metabolites, although Berry et al (1975) have shown that young nonpregnant rats fed the same test diet used in their prenatal studies excreted significant quantities of Phe, PPA, and o-hydroxyphenylacetic acid.

The present experimental model has satisfied the criterion of stable maternal hyperphe in the range associated with high risk to the human fetus. In addition, this objective was achieved with only moderate elevation of plasma Tyr as well as evidence of the excretion of some phenylketone. Guinea pigs fed the optimum test diet remained healthy, gained weight as food intake approached normal, and were able to successfully conceive (Series C, Series E, Experiment 2). Having acheived this primary goal, it became

possible to study the effects of hyperphe on pregnancy in guinea pigs.

Since pregnant PKU women have elevated Phe levels throughout pregnancy, it is important that experimental studies of maternal hyperphe include the early stages of pregnancy. In addition, the importance of studying the first part of pregnancy is highlighted by the alarming frequency of congenital malformations found among the offspring of women with Phe levels of 15 mg/100 ml or more (Table 6). Most studies of experimental maternal hyperphe have been limited to the latter half of pregnancy and in the few reports in which maternal hyperphe was induced during early pregnancy, the animals received only Phe administration therefore leading to confounding hypertyrosinemia (Boggs and Waisman 1962; Waisman et al 1964; Kerr and Waisman 1966; 1967; Waisman 1968; Carver et al 1965; Thompson and Kano 1965; Polidora 1967; Luse et al 1970; Kerr et al 1968; Chamove and Davenport 1970; Chamove et al 1973; Auerbach et al 1958; Ammon 1961; Wassmuth 1958). As has already been discussed, all of these studies have procedural shortcomings which make objective evaluation and interpretation difficult. The present study is the first to induce hyperphe with combined Phe and pCPA administration during the first half of pregnancy. The initial experiments in which maternal hyperphe was induced in guinea pigs suggested that both pCPA administration and very poor maternal

food intake may lead to abortion (Series E, Experiment 2). Although many workers have administered pCPA to pregnant animals the only other evidence to suggest that pCPA may be toxic to embryos was recently reported by Moore and Hampton (1974) working with rats. In Moore and Hampton's studies, unlike the present work, the pregnant animals suffered severe weight loss during the entire pCPA treatment period. In addition, some live embryos were carried to term in their experiments, whereas all of the guinea pigs allowed to continue consuming pCPA test diets aborted (Series E, Experiment 2). Moore and Hampton did not detect any gross malformations in the pCPA-treated fetuses nor did they measure maternal or fetal Phe levels. They were able however, to reduce the incidence of embryo death by decreasing the pCPA dosage during the first 12 days of gestation. The results of both Moore and Hampton's study and the current work demonstrate the necessity of pCPA control groups in studies in which both pCPA and Phe are used to induce hyperphe in pregnant animals.

Since early pregnancy loss and congenital malformations have been reported in maternal PKU and the understanding of the effects of pCPA on early development is limited, a detailed study of maternal hyperphe and pCPA treatment during early pregnancy in guinea pigs was undertaken (Series E, Experiment 3). These studies, which included a 3.5% Phe 1.0% pCPA hyperphe group as well as both 1.0% pCPA and 3.5% Phe

control groups, confirmed that embryo death was associated exclusively with maternal pCPA treatment. The mechanism of this effect of pCPA is unclear, although a number of possible explanations can be considered. The significant association between maternal plasma PA levels, but not maternal Phe levels as determined by paper chromatography, and the clearly dose related embryonic mortality reported by Moore and Hampton (1974), suggest that embryo death in the guinea pig may also be related to the dosage of pCPA. Both pCPA and Phe appear to be actively transported to the guinea pig embryo by at least gestation day 17 and this could facilitate a direct action of pCPA on the embryo (Figure 29). The role of hyperphe, in the absence of pCPA, is unlikely to be large since no embryonic mortality was observed among the Phe controls even though maternal Phe levels were elevated in the range of 4 to 7 mg/100 ml. In addition, the highest frequency of embryonic death was found in the 1.0% pCPA group which had at most, very moderate hyperphe (significantly less than the 3.5% Phe controls).

In addition to inhibition of Phe hydroxylase, pCPA has been found to have numerous behavioral and physiological effects in nonpregnant animals (eg: McGeer and McGeer 1973) and is also a potent inhibitor of tryptophan hydroxylase (Koe and Weisman 1966; Koe 1971). The inhibition of tryptophan hydroxylase by pCPA results in the lowering of tissue serotonin levels. The role of serotonin depletion in early

embryonic death however, is not known. Moore and Hampton (1974) have shown that at term, serotonin is significantly lowered in both fetal and maternal rat tissues following maternal pCPA treatment during pregnancy, although these workers did not present similar data from the stage of pregnancy associated with fetal death when high doses of pCPA were administered. Recent studies have shown that serotonin is present very early in embryonic life and that it may function as a growth regulator (eg: Ahmad and Zamenhof 1978). Embryonic death could certainly be caused by alterations in the levels and turnover of a substance required for early embryonic growth. If pCPA could be shown to cause embryo death by this type of mechanism, maternal pCPA administration might prove to be a valuable tool for further study of the role of serotonin in early development.

pCPA does not inhibit overall protein synthesis, although the synthesis of Phe hydroxylase in liver may be decreased following pCPA administration (Miller et al 1976). There is also evidence that pCPA may be incorporated into the tryptophan and Phe hydroxylase proteins (Gal et al 1970; Gal and Millard 1971) and if pCPA were incorporated into critical embryonic proteins and/or inhibited specific embryonic protein synthesis, either of these effects could irreparably damage the developing embryo. It is of note that many of the dead embryos in the present study were found to be grossly malformed and it is possible that malformation may

precede embryonic death in many instances. Further studies in which embryos are studied at various developmental stages and critical periods defined could determine whether gross malformation is the mechanism underlying pCPA-induced embryonic death.

pCPA might also interfere with the transport of other amino acids in the embryo, particularly Phe and tryptophan, which in turn could affect protein synthesis and disrupt normal development (Lines and Waisman 1970; Koe 1971; Knapp and Mandell 1972). It is also possible that pCPA and/or its metabolites have other noxious effects on embryonic cells. Since pCPA will continue to be used as a tool to study both hyperphe and serotonin during early development, it is important that future work clarify the embryotoxic effects of pCPA.

Although abortion has been reported in pregnant hyperphe women (Tables 1 through 6), the high frequency of abortion in the guinea pigs in the present study is clearly associated with pCPA and not maternal hyperphe. The incidence of congenital malformation however, is also increased among the children of hyperphe women (Tables 1 through 6) and early embryonic malformations were found in the embryos of hyperphe guinea pigs even in the absence of pCPA administration (Series E, Experiment 3). A statistically significant association between maternal plasma Phe levels and the frequency of abnormal embryos was found and in

addition, nearly one half of the embryos in the 3.5% Phe control group were malformed. Yolk sac Phe levels were consistently higher than maternal plasma Phe levels suggesting active transport of Phe to the early guinea pig embryo (Figure 29), a phenomenon which may be detrimental to the early embryo in the presence of maternal hyperphe. Although comparable data are not available in man, the high incidence of congenital malformations among the offspring of hyperphe women might be related to active transport of excessive amounts of Phe to the early embryo. The high frequency of malformed embryos in the 3.5% Phe group cannot be explained on the basis of poor food intake, insufficient weight gain or ascorbic acid deficiency, since this group did not differ significantly from either the Asp or Purina controls on any of these parameters. The possibility does remain that the embryonic abnormalities observed in the 3.5% Phe group may be due in part to high maternal or embryonic Tyr levels, although no statistical association between maternal plasma or yolk sac Tyr and embryonic abnormality was found. The possibility that the mild hyperphe (4 to 7 mg%) observed in the 3.5% Phe does indeed play a role in the genesis of embryonic malformation may have implications for maternal PKU. Concern has been raised about the increased frequency of congenital malformations among the children of hyperphe women, and the finding of malformations in guinea pigs with hyperphe does not allay that concern (Angeli et al

1974; Fisch et al 1969; Johnson 1972; MacCready and Levy 1972; Pueschel et al 1977). In this context it is important that the relationship between hyperphe and malformation in the guinea pig be clarified with respect to dose-response, critical periods, and the nature of malformations evident at term. The experimental literature does not include other reports of embryonic malformation attributable to maternal hyperphe and this clearly deserves further study.

In addition to offspring malformations, experimental models of maternal PKU should also determine whether in utero growth retardation is associated with maternal hyperphe. In the present study embryos were classified as developmentally retarded when at least two organ systems were found to have significantly delayed development. Evidence of developmental retardation was found among the embryos exposed to maternal Phe or pCPA treatment (Series E, Experiment 3), although no retarded embryos were found in the 3.5% Phe 1.0% pCPA group. Two litters in this group could not be assessed for embryonic retardation (Table 42) and it is therefore possible that delayed development occurred but was not detected. Since the number of somites, another indicator of the progress of early embryonic development, was decreased in the 3.5% Phe 1.0% pCPA group as well as the pCPA and Phe control groups, there does not appear to have been a protective effect with respect to developmental delay when Phe and pCPA administration was combined. The pCPA group was found to have both

the lowest somite number and the highest frequency of retarded embryos, suggesting that pCPA is a significant factor leading to the delayed development of early guinea pig embryos. Since a number of authors have presented evidence of intrauterine growth retardation in the offspring of rats given both Phe and pCPA, but have not included pCPA controls, the association of growth retardation with maternal hyperphe cannot be established in these studies (Schalock and Copenhaver 1973; Copenhaver et al 1974; Andersen 1976). Berry et al (1977) have reported decreased fetal brain weight in an appropriately controlled study of maternal hyperphe in rats. The reduced brain weight appears to be related to hyperphe, not pCPA, and could be reversed by feeding the pregnant hyperphe females additional valine, isoleucine and leucine. The protective effect of these branched chain amino acids, which also reversed the behavioral effects of maternal hyperphe, suggest that hyperphe may affect rat brain development by interacting with other amino acids in a critical but reversible manner. In the guinea pig however, both hyperphe and pCPA appear to play a role in retarding early embryonic development. Poor maternal food intake, ascorbic acid deficiency, or inadequate maternal weight gain did not appear to be directly related to the incidence of retarded embryos. Both the current study and Berry's work (Berry 1977) examined the effects of maternal treatment during part of the gestation period and both

approaches need to be extended to include the remainder of pregnancy. It would be useful to determine how the embryonic growth retardation associated with Phe treatment in the present study is manifested at term, for if it could be shown that maternal hyperphe during the first trimester affects embryonic and fetal growth, this could have implications for the timing of dietary treatment in maternal PKU.

Poor maternal food intake is a factor which has confounded many studies of experimental maternal hyperphe (eg: Boggs and Waisman 1962; Kerr and Waisman 1967; Kerr et al 1968; Luse et al 1970; Copenhaver et al 1970, 1973, 1974; Schalock and Copenhaver 1973; Andersen 1976). In the present work, both Phe and pCPA were found to be associated with decreasing food intake as their test diet content was increased (Series C, Experiments 1 and 2). Efforts to improve food intake by sweetening diets with sucrose and Asp were only partially effective (Series C, Experiment 5). Supplementation of test diets with Asp more effectively reduced poor food intake than did sucrose supplementation, and no deleterious effects could be attributed to Asp in either pregnant or nonpregnant animals. Poor maternal food intake however, was not found to be associated with embryonic mortality, abnormality, or retardation in Series E, Experiment 3. After gestation day 7, maternal food intake was nearly equal among the test diet groups so that it was primarly the relatively less vulnerable

An interesting observation in Series E, Experiment 3 was that yolk sac fluid levels of Phe and Tyr exceeded maternal plasma levels even in the presence of maternal hyperphe and hypertyrosinemia. These data indicate that amino acids are actively transported to the embryo at this early stage of development (equivalant to approximately 3 to 4 weeks post conception in man). Although comparable data are not available from human pregnancies, amniotic fluid amino acids have been measured at later stages. Cockburn et al (1970, 1972) have shown that in normal human pregnancy amniotic fluid Phe and Tyr are less than corresponding maternal plasma levels between 15 and 18 weeks of amenorrhea. Dallaire et al (1974) have shown that the Phe and Tyr concentration of human amniotic fluid decreases from 10 weeks gestation to term whereas maternal plasma levels do not decline during this period. It is not known how early in gestation this pattern of change begins, although it may be that very early in pregnancy amniotic fluid levels of Phe and Tyr might also exceed maternal plasma levels in man. It has also been shown that in human amniotic fluid Phe concentration is increased following maternal Phe loads as well as in maternal PKU (Cockburn et al 1972; Emery et al 1972). In these studies fetal plasma Phe and Tyr were greater than amniotic fluid levels. It was not possible to obtain embryonic blood for amino acid study in the present work to determine whether these levels may have

also exceeded yolk sac Phe and Tyr. During the last trimester of gestation however, fetal plasma Phe and Tyr were shown to exceed maternal levels in normal guinea pig pregnancy, indicating that active transport of amino acids to the conceptus occurs throughout most, if not all, of pregnancy in the guinea pig.

The experiments reported in Series D confirmed that in the presence of a Tyr load, in this situation via excess dietary Phe, the guinea pig requires ascorbic acid to efficiently catabolize Tyr (Zannoni and LaDue, 1960). In Series E, Experiment 3 maternal liver was assayed for ascorbic acid content and found not to differ significantly between groups. Under the conditions of that experiment, the oral ascorbic acid supplements were sufficient to prevent both hypertyrosinemia and ascorbic acid deficiency. It is of note that compared to similarly treated nonpregnant animals, the pregnant animals in Series E, Experiment 3 had different hepatic ascorbic acid levels; the pregnant Purina animals had lower levels and the pregnant 3.5% Phe 1.0% pCPA test diet animals higher levels (cf Series D, Experiment 2). Whether these differences are due to pregnancy itself, the duration of treatment, or the number of animals tested, is not known. It is clear however, that the deleterious effects on embryonic development observed in Series E, Experiment 3 cannot be attributed to ascorbic acid deficiency, nor can the abortions observed in Series E, Experiment 2

be related to inadequate maternal ascorbic acid intake. It is not clear why the oral ascorbic acid supplement given to the animals in Series D, Experiment 2 was associated with low hepatic ascorbic acid levels. The ascorbic acid supplement was considerably in excess of the requirements of both normal and physically stressed guinea pigs (Yew 1973; Yeen-Baigent et al 1975) and exceeded the daily vitamin C intake of the Purina control animals. It is unlikely that rapid excretion or lack of absorption of a large dose of the vitamin is responsible for the low hepatic levels observed, since animals given the daily dosage in two divided doses had significantly lower hepatic ascorbic acid levels than the animals given the same amount of ascorbic acid in a single administration. Since the ascorbic acid solution was freshly prepared and administered within minutes of preparation, it is extremely unlikely the animals actually received less than the 50 mg/300 g dose of the vitamin. It may be that animals maintained on the 3.5% Phe 1.0% pCPA diet in some way handle ascorbic acid differently than do animals fed Purina chow. Increased metabolic demand or different patterns of distribution might be factors.

The observation that approximately 10% of the female guinea pigs obtained from one supplier had unusually high plasma Tyr levels, even when consuming standard Purina chow, was an intriguing but unexpected finding (Appendix B). It is possible that some or all of these apparantly healthy

animals may have an inherited abnormality of Tyr metabolism. Since disorders of Tyr metabolism have been described in man (Berry 1976; Scriver and Rosenberg 1973) further characterization of the defect in these guinea pigs could be very informative.

In summary, the present study has demonstrated that stable maternal hyperphe can be induced in pregnant guinea pigs with only moderate elevations of Tyr. Evidence that pCPA is toxic to guinea pig embryos and that moderate maternal hyperphe may be associated with both embryonic malformation and developmental retardation has also been presented. The toxicity of pCPA limited the application of the present model, however newer agents which inhibit Phe hydroxylase may permit further use of the potentially informative guinea pig in studies of maternal hyperphe.

Appendix A

Introduction

Guroff has shown that rats have maximally reduced Phe hydroxylase activity for over 48 hours following a single injection of pCPA (Guroff 1969; Lipton et al 1967). Two experiments were therefore undertaken in which serum Phe, PA, and Tyr levels were measured in guinea pigs injected every 48 hours with pCPA and every 24 hours or every 12 hours with Phe.

Experiment 1

Eleven female guinea pigs were used. Eight Hartley strain guinea pigs, approximately 134 days of age, were obtained from A & E Farms. Three adult animals, of unknown age, were obtained from the stock of McMaster University Animal Facilities.

Three animals received Phe every 24 hours and pCPA every 48 hours. Three animals received pCPA every 48 hours and saline every alternate 24 hours. Three animals received Phe every 24 hours and an additional 2 animals received saline every 24 hours.

Both Phe and pCPA were injected IP at a dosage of 200 mg/kg. For injection Phe was dissolved in 0.85% saline and pCPA was suspended in 0.85% saline. Equal volumes of 0.85% saline (1 ml/100 g) were used for control injections. All animals were briefly stunned with CO_2 to facilitate the injections.

Serum was collected for fluorometric assay of PA, Phe and Tyr one week prior to the first injection and 3, 8, and 10 days after the injection treatments began. Blood samples were always collected just prior to the next scheduled injection. Blood was collected under methoxyflurane anesthesia (CO_2 induction, Hoar 1969) from the cephalic or jugular vein.

Each day urine was collected in metal trays placed below the cages and tested with $FeCl_3$ for the presence of phenylketones.

Daily food consumption and body weight were recorded.

Experiment 2

Ten of the animals used in Experiment 1 were used in this experiment. The procedure was identical to Experiment 1 except as noted below.

Four animals received pCPA every 48 hours and Phe every 12 hours. Three animals received pCPA every 48 hours and 0.85% saline every 12 hours, except at the times when pCPA was injected. Three animals were given Phe every 12 hours and one animal received 0.85% saline every 12 hours.

Serum was collected for fluorometric assays 2, 4, 6, and 12 days after the injections began. Blood samples were obtained approximately mid-way between the daily 12 hour injections.

Results

Experiment 1

Mean serum concentrations of Phe, PA, and Tyr are

summarized in Appendix A, Table 1. Animals treated daily with Phe and every 48 hours with pCPA had very small elevations of serum PA after 3 and 10 days of treatment. Animals treated with Phe, or pCPA, or saline, had similar serum Phe (or PA in the pCPA group) levels, which were not elevated above the pretreatment baseline levels. Tyrosine levels were not affected by any of these treatments.

Six (6) positive and one questionably positive urine $FeCl_3$ tests were found in the pCPA plus Phe group. Three questionably positive urines were found in animals treated only with pCPA. There was one positive sample from the animals injected with Phe only. All other urines had negative $FeCl_3$ tests.

In the 24 hours following blood collection, animals often lost weight, the average loss being 16.3 g (2.7% of first treatment day mean body weight). This weight loss was accompanied by a mean decrease in daily food consumption of 9.6 g (41.3% of the mean food consumption on the day preceding the first blood sample.). Both weight and food consumption usually returned to normal within 2 to 3 days following the blood collection.

Experiment 2

The mean serum Phe, PA and Tyr levels determined during Experiment 2 are summarized in Appendix A, Table 2. Comparison of these values with pretreatment Phe and Tyr levels

Appendix A, Table 1: Experiment #1

Mean (± S.D.) serum phenylalanine (or total phenylalanines) and tyrosine (mg/100 ml) during the experiment.

GROUP	PRE-TREATMENT		NUMBER OF DAYS OF INJECTIONS							
			3 DAYS		8 DAYS		10 DAYS		OVERALL	
(number of animals)	PHE (n)*	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)
Phe + pCPA	1.6 ±0.21	1.2 ±0.22	2.2	1.4	5.4	1.2	4.1	1.9	3.47 ±1.46	1.48 ±0.44
(3)	(5)	(5)	(3)	(3)	(1)	(3)	(3)	(3)	(7)	(9)
pCPA	1.7	1.3	2.1	2.0	3.3	2.0	1.7	1.4	2.25 ±0.89	1.86 ±0.83
(3)	(3)	(3)	(3)	(3)	(2)	(3)	(3)	(2)	(8)	(8)
Phe	1.8	1.0	3.0	2.4	1.7	1.3	2.8	1.5	2.50 ±1.22	1.65 ±0.51
(3)	(3)	(3)	(3)	(2)	(3)	(3)	(3)	(3)	(9)	(8)
Saline	N.D.+	N.D.	2.4	2.0	2.0	1.8	N.D.	N.D.	2.20 ±0.55	1.93 ±0.51
(2)			(2)	(2)	(2)	(2)			(4)	(4)
All	1.69 ±0.26	1.19 ±0.30								
(11)	(11)	(11)								

* number of samples analyzed

+N.D.: not determined

shows that the Phe and pCPA injection schedule did not produce significant hyperphe. In addition, there were no apparant differences in serum Phe (PA) or Tyr among the treatment groups.

Positive $FeCl_3$ tests were observed in 8 urines in the Phe plus pCPA group and in 5 urines in the pCPA group. No other urine samples had positive $FeCl_3$ tests.

As in Experiment 1, the animals usually lost weight and reduced food consumption during the 24 hours following blood collection. Both parameters returned to normal levels about 48 hours after blood collection.

Discussion

Injection of guinea pigs every 48 hours with pCPA and every 24 or every 12 hours with Phe did not produce sustained hyperphe. A number of workers have injected rats with similar regimens of pCPA and Phe and demonstrated significant hyperphe (and/or elevations of PA) during the 3 hours following Phe injections (eg: Lipton et al 1967; Andersen et al 1973; Hole 1972; Copenhaver et al 1973; Glazer and Weber 1971a; Andersen 1976). Most of these authors however, did not provide adequate data to determine the duration of the induced hyperphe. The present work suggests that although hyperphe may have been produced by the combined pCPA and Phe injections, it must have been of a transient nature, lasting less than 6 hours after the Phe injections.

All but one of the urine samples containing $FeCl_3$ positive material were from animals treated with pCPA, with

Appendix A, Table 2: Experiment #2

Mean (±S.D.) serum phenylalanine (or total phenlalanines) and tyrosine (mg/100 ml) during the experiment.

GROUP (number animals)	PRE-TREATMENT		NUMBER OF DAYS OF INJECTIONS									
			2 DAYS		4 DAYS		6 DAYS		12 DAYS		OVERALL	
	PHE (n)*	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)	PHE (n)	TYR (n)
Phe + pCPA	1.6 ±0.21	1.2 ±0.22	1.5	1.5	1.9	2.0	1.2	1.3	1.6	2.0	1.46 ±0.37	1.56 ±0.37
(4)	(5)	(5)	(3)	(2)	(1)	(1)	(3)	(3)	(1)	(1)	(8)	(7)
pCPA	1.7	1.0	2.0	1.3	1.5	1.6	1.8	1.6	1.2	1.9	1.66 ±0.35	1.55 ±0.32
(3)	(3)	(3)	(3)	(3)	(1)	(1)	(2)	(2)	(2)	(2)	(8)	(8)
Phe	2.0	1.3	2.0	1.8	N.D.	N.D.	1.7	1.5	1.2	1.9	1.68 ±0.46	1.69 ±0.45
(3)	(2)	(2)	(3)	(2)			(3)	(3)	(2)	(2)	(8)	(7)
Saline	N.D.	N.D.	1.3	0.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3	0.6
(1)			(1)	(1)							(1)	(1)
All	1.70 ±0.27	1.17 ±0.31										
(10)	(10)	(10)										

*(n) = number of samples analyzed

N.D. = not determined

or without additional Phe treatment. None of these animals had persistent hyperphe and the identity of the $FeCl_3$ positive substance therefore may not be PPA. Rats have been shown to transaminate pCPA to p-chlorophenylpyruvic acid (Koe 1971; Gal et al 1970), and this compound might also be excreted by pCPA-treated guinea pigs (Flatlow 1910, cited by Tong, et al 1973). This possibility is considered further in the experiments reported in Series B.

Since weight loss and diminished food intake followed both successful and unsuccessful attempts to collect blood, these effects must be primarily a result of surgical trauma rather than blood loss. In later work, methods of collecting blood were developed which were not associated with a weight loss or poor food intake.

Since inadequate hyperphe was obtained in these experiments, a more detailed study of the adult guinea pig's response to injections of Phe and pCPA was undertaken. These experiments are described in Series B.

Appendix B

Introduction

The normal plasma Tyr concentration of guinea pigs is usually less than 2.0 mg/100 ml. During the course of the present studies a number of apparantly healthy adult guinea pigs were unexpectedly found to have plasma Tyr levels which were well above this normal range. Since it is possible that these animals may have an inherited abnormality of Tyr metabolism, the plasma Phe and Tyr levels of these guinea pigs are included in this appendix.

Methods

All animals were female Hartley strain guinea pigs obtained from M. & L. Farms. Plasma was collected from the saphenous vein and assayed for Phe and Tyr in duplicate by the fluorometric or spectrophotometric methods. When sufficient plasma was available Phe and Tyr were also estimated by paper chromatography.

Results

Baseline plasma Phe and Tyr levels were routinely determined on animals used in the studies of hyperphe just described. The blood was collected while the animals were consuming either Purina chow or a diet supplemented only

with 0.025% Asp; those fed the Asp diet received oral ascorbic acid supplements. During the final year of this work over 100 guinea pigs were obtained from M. & L. Farms and 10 of these were found to have plasma Tyr greater than or equal to 3.0 mg/100 ml (Table). The mean Tyr concentration of those guinea pigs obtained during this period without hypertyrosinemia was 1.09 mg/100 ml and two standard deviations above the mean equalled 2.01 mg/100 ml. Plasma Tyr of the 10 animals with levels equal to or greater than 3.0 mg/100 ml ranged up to 35 mg/100 ml, and there was considerable variability both between animals and from day to day for each animal (Table). One animal (77A) for example, had a Tyr level of 5.0 mg/100 ml and a few days later the Tyr level was then found to be within normal range (Table). Many of these guinea pigs were also fed 3.5% Phe 1.0% pCPA test diets. While consuming this test diet these animals frequently had unusually high Tyr levels, occassionally exceeding 45 mg/100 ml. As indicated previously, animals with elevated baseline Tyr levels have been excluded from the data presented in the previous studies.

The baseline Phe levels of these animals were usually within two standard deviations of the mean (ie less than 2.31 mg/100 ml) although two of the guinea pigs did exceed this level (Table).

All of these animals appeared healthy and gained weight at appropriate rates. All were ascertained retro-

Appendix B Table 1. Plasma phenylalanine and tyrosine (mg/100 ml) in control guinea pigs and those found to have elevated tyrosine levels. All blood samples collected when animals were consuming either Purina chow or Purina chow plus 0.025% Aspartame.

Animal	Fluorometric/ spectrophotometric		Paper Chromatographic	
	Phe	Tyr	Phe	Tyr
6A*	1.8	5.7	< 6	< 4
10A	2.2	14.3	< 6	18
	2.1	13.5	—	—
20A	2.4	4.8	< 6	< 4
	2.5	4.4	< 6	4
23A	2.2	4.8	< 6	7
55A	1.2	3.8	< 6	4
74A	1.9	12.0	< 6	12
	1.5	5.0	< 6	< 4
77A	1.3	5.0	< 6	< 4
	1.3	1.2	< 6	4-5
91A	1.7	4.2	< 6	< 4
98A	2.2	3.0	< 6	< 4
6B	2.2	27.8	< 6	25
	3.0*	29.7	< 6	> 36
	3.2*	35.2	< 6	> 36
	3.1*	32.2	< 6	24
Controls (N = 82)	Mean ±S.D. 1.42 ±0.44	Mean ±S.D. 1.09 ±0.46	all < 6	all < 4

* pregnant at time blood sample collected.

spectively on the basis of high plasma Tyr concentration and not on the basis of any obvious difference from the other guinea pigs in the laboratory.

The prevelance of hypertyrosinemia, defined as Tyr levels greater than or equal to 3.0 mg/100 ml, among female guinea pigs from M. & L. Farms is estimated to be approximately 10 per 100 animals. This abnormality was not observed in any of the 73 animals obtained from other suppliers.

Discussion

It may be that some of these guinea pigs with unusually high plasma Tyr levels have inherited abnormalities of Tyr metabolism. The possibility that this characteristic may be genetically controlled is supported by the observation that animals from only one breeding colony were found to have high Tyr. It is possible that the 3 animals with Tyr levels exceeding 12 mg/100 ml may have a more severe block in Tyr metabolism than is present in the remaining 7 animals with levels between 3 and 5.7 mg/100 ml. Alternatively, the differences in Tyr elevation mg be related to the recency and magnitude of dietary Tyr ingestion prior to the time of blood collection. Tyr intake was clearly related to plasma Tyr levels as shown by the very much higher Tyr levels these animals had when consuming Phe supplemented test diets. Studies of both the enzymes and metabolites involved in Tyr catabolism would be valuable to clarify the underlying nature of this abnormality. In addition, breeding experiments could

be undertaken to determine the mode of inheritance of this intriguing characteristic.

A number of patients have been described with presumed disorders of Tyr metabolism (Berry 1976; Scriver and Rosenberg 1973). The possibility that some of these guines pigs might have similar disorders is clearly worthy of further investigation.

Appendix C. Abbreviations used in text.

ANOVA: analysis of variance

Asp: Aspartame (L-aspartyl-L-phenylalanine methylester)

DMPTH: dimethyltetrahydropteridine

$FeCl_3$: ferric chloride

g: gram

G: gravity

hyperphe: hyperphenylalaninemia

IP: intraperitoneal

NADH: reduced nicotinamide adenine dinucleotide

PA: total phenylalanines (phenylalanine + p-chlorophenylalanine, as measured by the fluorometric assay.)

pCPA: D, L-para-chlorophenylalanine

Phe: L-phenylalanine

PKU: phenylketonuria

PPA: phenylpyruvic acid

S.D.: standard deviation

suc: sucrose

TCA: trichloroacetic acid

Tyr: L-tyrosine

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