

METABOLIC EFFECTS OF CAFFEINE ON CHICKS AND MICE

by

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TABLE OF CONTENTS

| | Page |
|--|------|
| LIST OF TABLES | v |
| LIST OF ILLUSTRATIONS | vi |
| ABSTRACT | vii |
| 1. INTRODUCTION AND REVIEW OF LITERATURE | 1 |
| Review of Literature | 2 |
| Caffeine Metabolism and Distribution | 2 |
| Genetics | 3 |
| Reproduction | 3 |
| Effects on Blood Glucose | 4 |
| Effects on Plasma Lipids | 5 |
| Growth Studies | 5 |
| 2. DIETARY CAFFEINE STUDIES WITH MICE | 7 |
| Experimental Procedure | 7 |
| Results and Discussion | 9 |
| Summary | 22 |
| 3. DIETARY CAFFEINE STUDIES WITH CHICKS | 24 |
| Experimental Procedure | 24 |
| Results and Discussion | 26 |
| Summary | 34 |
| REFERENCES | 38 |

LIST OF TABLES

| Table | Page |
|--|------|
| 1. Basal Diet Composition for Both Mouse Experiments | 8 |
| 2. Effect of Caffeine on the Growth of Young Mice (M-1) | 10 |
| 3. Effect of Caffeine on Dietary Nutrient Absorption (M-1) | 12 |
| 4. Glutamic Oxalacetic Transaminase Activity in Response to Caffeine in Mice (M-1) | 13 |
| 5. Effect of Caffeine on the Growth of Young Mice (M-2) | 14 |
| 6. Effect of Caffeine on Dietary Nutrient Absorption (M-2) | 19 |
| 7. Tissue Enzymatic Activity in Response to Dietary Caffeine in Mice (M-2) | 21 |
| 8. Basal Diet Composition for Both Chick Experiments | 25 |
| 9. Effect of Caffeine on Body Weights and Feed Utilization of Growing Chicks (C-1) | 27 |
| 10. Effect of Caffeine on the Retention of Dietary Nutrients (C-1) | 29 |
| 11. Effect of Caffeine on Body Weights and Feed Utilization of Growing Chicks (C-2) | 30 |
| 12. Effect of Caffeine on the Retention of Some Dietary Nutrients (C-2) | 35 |
| 13. Liver Glutamic Oxalacetic Transaminase Activity in Response to Dietary Caffeine in Chicks (C-2) | 36 |

LIST OF ILLUSTRATIONS

| Figure | Page |
|---|------|
| 1. Effect of Dietary Level on Caffeine Consumption of Mice | 16 |
| 2. Effect of Caffeine Ingestion on Feed Consumption of Mice | 18 |
| 3. Effect of Dietary Level on Caffeine Consumption of Chicks | 32 |
| 4. Effect of Caffeine Ingestion on Feed Consumption of Chicks | 33 |

ABSTRACT

Four experiments were conducted with weanling mice and day old chicks to determine the effects of dietary caffeine on growth, nutrient utilization and tissue enzymatic activity for a period of four weeks. In two experiments, the growth of mice fed diets containing caffeine levels from 370 to 24200 ppm showed statistical depressions at 1500 ppm and greater. A level of 4000 ppm affected mortality and the rate increased with caffeine concentration until 100% mortality was obtained at 12,100 and 24,200 ppm. Calcium, protein, and fat digestions were not affected by caffeine but phosphorus absorptions were decreased at 2000 to 6070 ppm. Liver xanthine oxidase activity was unaltered by the various caffeine treatments. Glutamic oxalacetic transaminase activity of kidney homogenates showed significant elevation at 6000 and 6070 ppm whereas the increased enzymatic activity of liver tissue was not found to be significantly different.

Chicks were fed diets consisting of 55 to 3550 ppm caffeine. Body weights were depressed at 888 ppm and above. Nitrogen retentions were reduced at 3000 to 3550 ppm whereas calcium, phosphorus, and fat retentions were unaltered by caffeine. There were no significant differences in glutamic oxalacetic transaminase activity of liver homogenates due to caffeine supplementation.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

The consumption of caffeine, as a component of popular beverages, dates from ancient times. This alkaloid is ingested as a constituent of coffee and cola flavored drinks and is ingested with two other xanthine derivatives--theobromine from cocoa and theophylline from tea.

Pharmacologic effects of these alkaloids are well defined. Medicinally, they are used as stimulants for cardiac muscle, skeletal muscle and central nervous system; they are also employed as milk diuretics (The Merck Index 1968).

One gm. of caffeine may be toxic to humans; the lethal dose is approximately 10 gm. (Clarke 1969). As one cup of instant coffee provides 80-100 mg. caffeine and one cup of strong percolator coffee furnishes up to 150 mg. caffeine (Wolman 1955), the habitual coffee drinker can consume a toxic amount of this alkaloid. Toxicity may be enhanced by the availability of caffeine in other popular drinks, in pharmaceutical preparations as No-Doz and in combination with other drugs as analgesic mixtures.

Despite the large consumption of caffeine as a food constituent, and its therapeutic value, reports concerning physiological and psychological effects are often contradictory. Conflicting results have been obtained from research, investigating the relationship of caffeine to genetics, reproduction, glucose and lipid metabolism.

Review of Literature

Caffeine Metabolism and Distribution

Caffeine was administered to two human subjects in a one gram dose and 66% of it was recovered in the urine during the following 48 hours, in equal amounts of methyluric acids and methylxanthines (Cornish and Christman 1957). Metabolites found were 1,7-dimethylxanthine, 7-methylxanthine, 1-methylxanthine, 1-methyluric acid, and 1,3-dimethyluric acid. Demethylation of caffeine (1, 3, 7 trimethylxanthine) may occur in order of increasing stability at methyl groups 3, 7, and 1. Apparently, demethylation of caffeine, theobromine, and theophylline ceases with monomethylxanthines as evidenced by no accumulation of xanthine in the urine and negligible increase in uric acid excretion.

Caffeine is metabolized rapidly and almost completely in man as only 1% was excreted unchanged in a 24 hour urine sample, after 500 mg. of caffeine was administered by means of intravenous injection (Axelrod and Reichenthal 1953). Plasma peak levels of caffeine occurred one hour after either oral or intravenous administration of 7 mg./kg. The bio-half life was estimated to be 3.5 hours for man and 5 hours for dogs. Even with continuous ingestion, limited day to day accumulation of caffeine in man was determined by its disappearance from the plasma, the morning following coffee consumption.

Caffeine distribution in the tissues is proportional to their water content and passes freely across all cellular membranes. Goldstein and Warren (1962) found caffeine in the same concentration in human

ovaries, testes, and plasma, implying potential genetic damage for habitual coffee drinkers.

Genetics

The mutagenicity of caffeine has been established in *E. Coli* (Koch 1956), *Drosophila* and human cells in vitro (Kuhlmann et al. 1968). The principle of dominant lethal mutation, as an indicator of genetic damage, has been employed by various researchers to obtain evidence of the mutagenic effect of caffeine on mammals in vivo. Epstein et al. (1970) treated male mice with caffeine by various forms of administration in doses up to 240 mg./kg. for acute studies and in smaller amounts for longer term studies. Subsequent mating of these mice and autopsy of mated females were employed to assay dominant lethal mutations (determined by the number of pre-implantation losses and early foetal deaths). There was no relationship between caffeine and mutagenic effects; however, pregnancy rate was significantly reduced in females mated to test animals. Confirmation of the failure of caffeine to induce dominant lethal mutations was reported by Adler (1969), who treated male mice with a single intraperitoneal dose of 250 mg./kg. Kuhlmann et al. (1968) reported that caffeine, added to drinking water at a concentration of 0.025 to 0.5%, gave evidence of mutagenicity in male mice.

Reproduction

The consumption of 40 to 50 mg. caffeine/kg./day by albino rats for 3 1/2 years (Bachmann et al. 1946) resulted in the normal number of litters and normal offspring. Epstein et al. (1970), as previously

reported, found a reduction in pregnancy rate in female mice mated to males that had been treated with doses up to 240 mg. caffeine/kg. In addition, Nishimura and Nakai (1960) showed that a single intraperitoneal dose of 250 mg. caffeine/kg. to pregnant mice caused death and malformations of the skeletal system of the embryo.

Effects on Blood Glucose

The effect of caffeine on blood glucose level is confusing, as published studies report both elevation and depression. Wachman et al. (1970) and Cheraskin et al. (1967) showed an increase in blood glucose concentration in human subjects for up to 2 hours after ingestion of 250 mg. caffeine. A relationship between human diabetes and caffeine intake was suggested (Kuftinec and Mayer 1964) when an elevation in blood glucose was found in obese hyperglycemic mice after injection with 2 mg. caffeine. When lean animals were injected with 5 mg. caffeine, a 24 hour decrease in blood glucose occurred, followed by a return to normal. Consumption of 875 mg. caffeine/day for 20 days (Naismith et al. 1970) showed no correlation between caffeine ingestion and blood glucose, insulin, or plasma lipids in human subjects.

Feinberg et al. (1968) reported hypoglycemia with a concomitant rise in serum free fatty acid (FFA) level after human subjects ingested 220 mg. caffeine with 1 gm. glucose/kg. It was determined previously that caffeine blocks the inactivation of cyclic AMP (in vitro by Sutherland) and that this cyclic nucleotide promotes the conversion of liver glycogen to blood sugar (glycogenolysis). The mechanism to

to account for the hypoglycemia found by Feinberg et al. (1968) is not established.

Effects on Plasma Lipids

Although Naismith et al. (1970) indicated plasma lipids to be unaltered by caffeine consumption in humans, other available reports showed an increase in lipid fractions. Data from a study by Naismith, Akinyanju, and Yudkin (1969) with rats gave evidence of an increase in plasma cholesterol and phospholipid concentration. Bellet, Kershbaum, and Finck (1968) found a rise in plasma free fatty acids after human consumption of 250 mg. caffeine. The mechanism for the increase in FFA by caffeine is not clearly defined; the only evidence is that caffeine increases catecholamines (Bellet et al. 1969) which in turn may affect plasma free fatty acid levels.

Growth Studies

There are few published reports pertaining to the nutritional aspects of caffeine. Chase (1928) force fed doses of caffeine ranging from 94 mg./kg. to 150 mg./kg. to chicks for 90 days. A decrease in growth and a slight loss of appetite resulted.

Bachmann et al. (1946) investigated the effects of caffeine on the growth of albino rats for a 3 1/2 year duration. Caffeine, supplemented to drinking water, was consumed at a level of 40 to 50 mg./kg./day. The rate of growth of the test animals was unaltered.

Inhibited growth of female albino rats was observed when doses greater than 150 mg. caffeine/kg./day were administered by intragastric

cannula for 100 days (Boyd et al. 1965). There was a high correlation between the percentage of growth inhibition and increase in dosage. Feed consumption was decreased only for one week preceding death. There was no correlation between inhibition of food intake and increase in dosage.

Naismith et al. (1969) reported an initial reduction in growth rate and feed consumption of male rats fed a starch-based diet supplemented with caffeine (140 mg./100 gm. diet) for 54 days. After the fourth day, no further reductions were observed. The dietary intake corresponded to 12 cups/day by a 70 kg. man.

Because of the limited data available, this thesis consists of experiments designed to analyze the effects of caffeine on growth, utilization of some dietary nutrients and some tissue enzymatic activity.

CHAPTER 2

DIETARY CAFFEINE STUDIES WITH MICE

Experimental Procedure

Weanling Charles River mice (U. of A. stock), weighing an average of 9 gms., were randomly selected and distributed among two experiments for a period of four weeks each. Twelve mice (6 males and 6 females) were assigned to each treatment with two mice of the same sex being housed together in each raised wire screen cage. Powdered feed, in feeders with wire screens to prevent wastage, and distilled water were supplied ad libitum for the duration of each experiment.

In the first experiment (M-1), caffeine, from Matheson, Coleman and Bell Co. was added to the basal diet (Table 1) to provide eight treatments consisting of 0, 370, 770, 1500, 3030, 6070, 12,100, and 24,200 ppm caffeine. The basal diet for experiment M-2 was supplemented with caffeine, furnishing six treatments containing 0, 2000, 3000, 4000, 5000, and 6000 ppm caffeine. Chromium oxide was added to the diets as an index for the determination of dietary nutrient absorptions.

Data including body weights and feed consumption for the third and fourth weeks plus mortality data for the four weeks were recorded. Feces samples were collected and refrigerated for the entire experimental period.

At the termination of both experiments, 10 animals per treatment were sacrificed by decapitation (with the exception of experiment M-1,

Table 1. Basal Diet Composition for Both Mouse Experiments

| Ingredient | Percent of Diet Exp. M-1 | Percent of Diet Exp. M-2 |
|-----------------------------------|--------------------------------|--------------------------------|
| Whole egg | 23.00 | 23.00 |
| Cerelose | 53.81 | 53.81 |
| Salt | 0.20 | 0.20 |
| Cellulose | 3.00 | 3.00 |
| Trace mineral mix ¹ | 0.20 | 0.20 |
| Vitamin mix purified ² | 4.00 | 4.00 |
| Corn oil | 1.00 | 2.00 |
| Calcium carbonate | 1.84 | 0.42 |
| Dicalcium phosphate | - | 1.96 |
| Sodium phosphate mono | 0.73 | - |
| Choline chloride | 0.20 | 0.20 |
| Chromium oxide | 0.20 | 0.20 |
| Bentonite | <u>11.82</u> | <u>11.01</u> |
| Total | 100.00 | 100.00 |

- Supplied as mg./kg. (ppm) of diet: iron 20, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; zinc 60, ZnO ; molybdenum 1, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; manganese 60, MnO_2 ; calcium 168, CaCO_3 ; copper 4, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; iodine 1.5, KI; and cobalt 1.5, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ in glucose monohydrate carrier.
- Supplied the following per kg. of diet: 10,012 I.U. vitamin A palmitate, 961 I.C.U. vitamin D_3 , 8.7 I.U. d-alpha-tocopheryl acetate, 30 mcg. vitamin B_{12} , 6.6 mg. 2-methyl-naphthoquinone, 12 mg. riboflavin, 88.1 mg. niacin, 15.2 mg. d-calcium pantothenate, 4 mg. thiamine hydrochloride, 0.9 mg. folic acid, 1.8 mg. pyridoxine, 91 mcg. biotin, 50 mg. ethoxyquin.

treatment 6, in which case only the five surviving mice were utilized). Livers and kidneys were excised and frozen immediately with dry ice. Tissues were stored at -4°C until homogenates were analyzed for the activity of xanthine oxidase (Horecker and Heppel 1949) and glutamic oxalacetic transaminase (Sigma 1961). Changes in optical densities were recorded from a Coleman spectrophotometer.

Feed and feces were analyzed for Cr_2O_3 (Czarnocki, Sibbald, and Evans 1961), calcium by flame photometry (Coleman 1958), phosphorus (Koenig and Johnson 1942), protein by the Kjeldahl method and fat (Goldfinch extraction). Data were analyzed statistically by analysis of variance and Duncan's multiple range test (Duncan 1955).

Results and Discussion

Data from the first study show a significant decrease in the fourth week body weight of mice fed 1500 ppm caffeine (Table 2). Growth was reduced further at the 3030 to 6070 levels. The feeding of 12,100 ppm caffeine caused 100% mortality by day 11, and 24,200 ppm caused 100% mortality by day 2. A 58% mortality rate occurred in mice fed 6070 ppm caffeine, whereas levels of 3030 ppm or less produced no deaths.

The effect of dietary caffeine level on fourth week body weight was determined by calculation of the regression line, using data from Table 2. Equation for the line: $Y = -.0027X + 25.7289$. The correlation coefficient, $r = -.9412$, is highly significant ($P = .01$) and dietary level is a good predictor for body weight. The negative

Table 2. Effect of Caffeine on the Growth of Young Mice (M-1)

| Dietary Treatments (ppm added) | 4th Week Body Weight (gms.) | Feed Consumed Mouse/Week (gms.) | Calculated Consumption of Caffeine Mouse/Week (mg.) | % Mortality |
|--------------------------------|-----------------------------|---------------------------------|---|-------------|
| 0 | 25.6 ^{c1} | 28 ^b | 0 | 0 |
| 370 | 26.1 ^c | 29 ^b | 11 ^a | 0 |
| 770 | 25.5 ^c | 28 ^b | 22 ^{ab} | 0 |
| 1500 | 20.6 ^b | 24 ^b | 36 ^b | 0 |
| 3030 | 13.9 ^a | 13 ^a | 37 ^b | 0 |
| 6070 | 11.4 ^a | 11 ^a | 67 ^c | 58 |
| 12100 | - | - | - | 100 |
| 24200 | - | - | - | 100 |

1. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

regression coefficient and correlation coefficient indicate the inverse relationship between caffeine level and body weight.

There was a significant decrease in the feed consumption of mice at 3030 ppm caffeine and a reduction, although not significant at 1500 ppm.

The amount of caffeine ingested increased significantly at 1500 ppm and a further increase was noted at 6070 ppm. Only five mice survived treatment 6 (6070 ppm) and the within treatment variability found in caffeine consumption makes the value of the mean consumption for the treatment questionable. Caffeine consumption appeared to plateau at 1500 to 3030 ppm, coinciding with a large decrease in weight and feed intake.

Increasing levels of dietary caffeine had no effect on calcium, nitrogen or fat absorptions, as shown in Table 3. High levels (3030 and 6070 ppm) appeared to reduce the amount of phosphorus absorbed.

The activity of liver glutamic oxalacetic transaminase (Table 4) was significantly elevated in mice fed 770 to 3030 ppm caffeine. An increase in activity over that of the control group occurred in mice fed 370 and 6070 ppm, but this was not significant. Glutamic oxalacetic transaminase activity of kidney homogenates showed a statistical increase at 6070 ppm compared with the controls and the groups on 370 and 1500 ppm caffeine.

In the second experiment, progressive significant depressions in growth rate occurred in mice fed dietary treatments of 2000, 4000, and 6000 ppm caffeine (Table 5). A 30% decrease in body weight resulted in

Table 3. Effect of Caffeine on Dietary Nutrient Absorption (M-1)

| Dietary Treatments (ppm added) | % Calcium Absorption | % Phosphorus Absorption | % Nitrogen Absorption | % Fat Absorption |
|--------------------------------------|----------------------------|-------------------------------|-----------------------------|------------------------|
| 0 | 25.07 | 54.75 | 83.39 | 94.87 |
| 370 | 23.19 | 62.95 | 84.11 | 94.96 |
| 770 | 26.04 | 54.78 | 84.37 | 94.75 |
| 1500 | 25.10 | 65.66 | 84.38 | 94.74 |
| 3030 | 22.16 | 43.52 | 84.08 | 94.51 |
| 6070 | 26.50 | 29.96 | 83.52 | 95.17 |

Table 4. Glutamic Oxalacetic Transaminase Activity¹ in Response to Caffeine in Mice (M-1)

| Dietary Treatments (ppm) | Tissue | |
|--------------------------------|---------------------|---------------------|
| | Liver | Kidney |
| 0 | 51.72 ^{a2} | 63.42 ^a |
| 370 | 64.49 ^{ab} | 67.77 ^a |
| 770 | 75.09 ^b | 70.97 ^{ab} |
| 1500 | 72.22 ^b | 65.20 ^a |
| 3030 | 75.04 ^b | 73.32 ^{ab} |
| 6070 | 68.70 ^{ab} | 84.70 ^b |

1. Enzymatic activity is expressed as change in O.D./min./gm. protein. Protein is measured at 280 m μ .
2. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

Table 5. Effect of Caffeine on the Growth of Young Mice (M-2)

| Dietary Treatments (ppm added) | 4th Week Body Weight (gms.) | Feed Consumed Mouse/Week (gms.) | Calculated Consumption of Caffeine Mouse/Week (mg.) | % Mortality |
|--------------------------------|-----------------------------|---------------------------------|---|-------------|
| 0 | 26.3 ^{d1} | 27 ^d | 0 | 0 |
| 2000 | 18.3 ^c | 17 ^c | 34 ^a | 0 |
| 3000 | 16.6 ^c | 15 ^c | 46 ^a | 0 |
| 4000 | 12.2 ^b | 11 ^b | 43 ^a | 8 |
| 5000 | 10.8 ^{ab} | 8 ^{ab} | 37 ^a | 17 |
| 6000 | 8.9 ^a | 6 ^a | 35 ^a | 17 |

1. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

mice fed 2000 ppm, compared with that of the control group. At 4000 ppm, a 54% reduction in growth rate was observed and at 6000 ppm, a 66% difference in weight was found.

The relationship of growth to dietary caffeine concentration was determined by computation of linear regression. The regression equation: $Y = -.0029X + 25.1928$. The highly significant correlation coefficient, $r = -.9860$ ($P = .01$), indicates a decline in body weight as a result of caffeine increments.

Dietary caffeine produced 8% mortality at a level of 4000 ppm and 17% mortality at both 5000 and 6000 ppm. At 3000 ppm or less, the mortality rate was zero. In this experiment, the mice tolerated a larger quantity of caffeine than that reported in the literature. The mice fed 6000 ppm incurred the 17% mortality rate before the collection of consumption data for the third and fourth weeks. The daily consumption for this group, determined from the fourth week data, was 562 mg./kg. Kuftinec and Mayer (1964) reported the LD_{50} under conditions of ad lib feeding to be 400 mg./kg. for lean mice.

Feed intake was significantly reduced in mice fed diets containing 2000, 4000, and 6000 ppm caffeine. Consumption of caffeine was not statistically altered in mice that ingested 2000 to 6000 ppm, implying a plateau of intake at these dietary levels.

Further examination of the plateau effect was performed by linear regression of caffeine consumption on dietary level, as illustrated in Figure 1. Data points are from both experiments with the omission of treatment 6, experiment 1, due to the large within

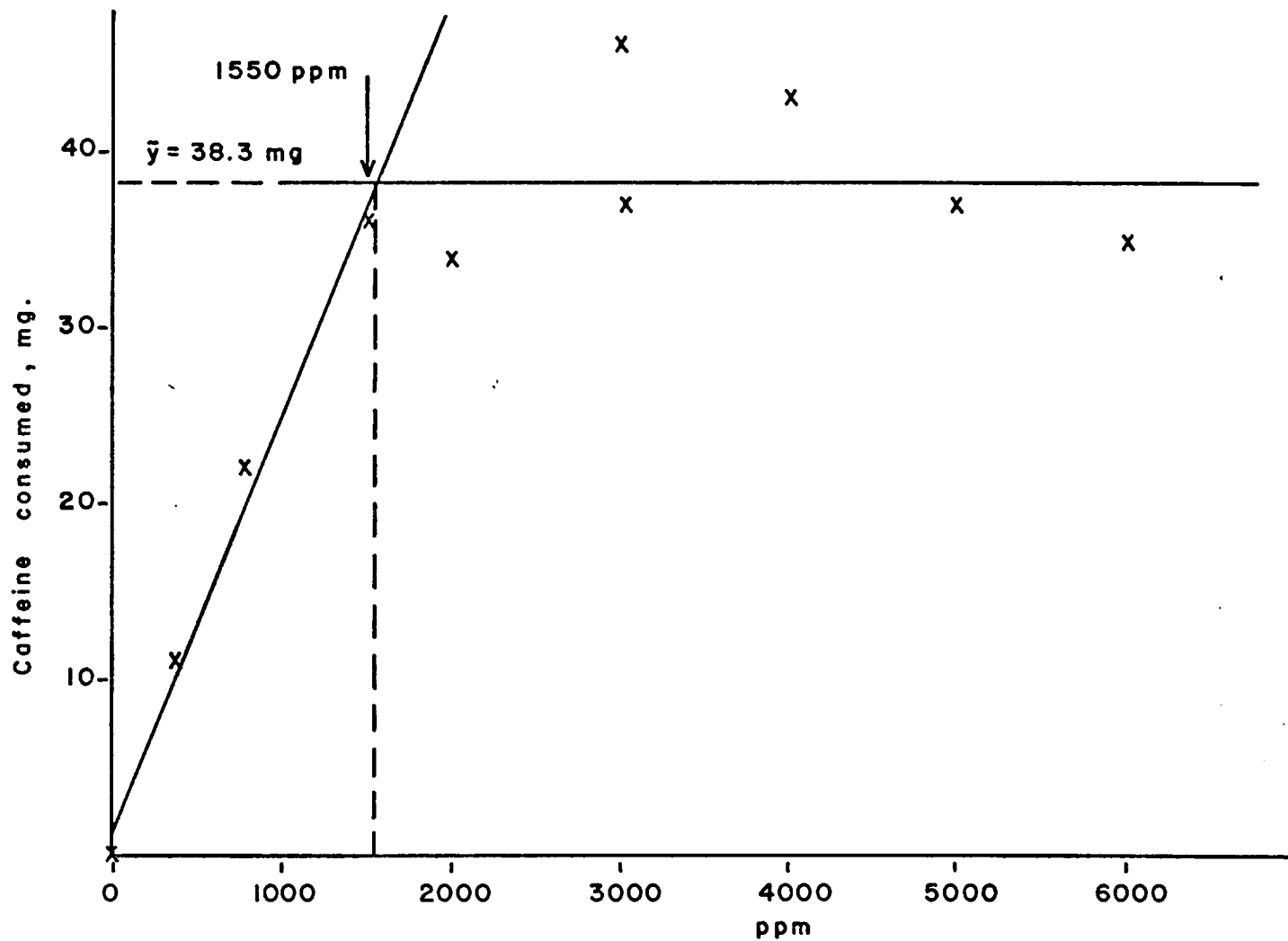


Figure 1. Effect of Dietary Level on Caffeine Consumption of Mice

treatment variability in caffeine consumption. The calculated correlation coefficient for the first equation, $r = .9937$, is highly significant at the .01 probability level and indicates a linear increase in caffeine consumption with increments of dietary levels to 1550 ppm, the point of intersection. A plateau is demonstrated by the second line as the correlation coefficient, $r = .0009$, indicates dietary levels of 1550 ppm to 6000 ppm have no effect on caffeine ingestion. In this case, the consumption of caffeine leveled off at 38.3 mg., the value for the mean intake.

This plateau implies that caffeine ingestion regulates feed consumption. The regression of feed consumption on caffeine intake is shown in Figure 2 and includes data from both experiments, with the exclusion of treatment 6, experiment 1. The first line is interpreted as no linear relationship between feed intake and caffeine consumption, as the correlation coefficient, $r = .4264$. Intersection of the regression lines would indicate that feed consumption begins to decline at 15 mg. caffeine ingestion. However, the correlation for the second line is not meaningful, as $r = -.5696$ (compared with tabular $r = .707$ at $p = .05$ and 6 error d.f.) and the amount of caffeine ingested apparently has little effect on feed consumption, as determined by linear regression.

Calcium, nitrogen and fat absorptions were unaltered by supplements of caffeine, as shown in Table 6. Phosphorus absorption was generally reduced with increasing levels of caffeine up to 6000 ppm where it was decreased compared with the control group and the mice fed 2000 ppm.

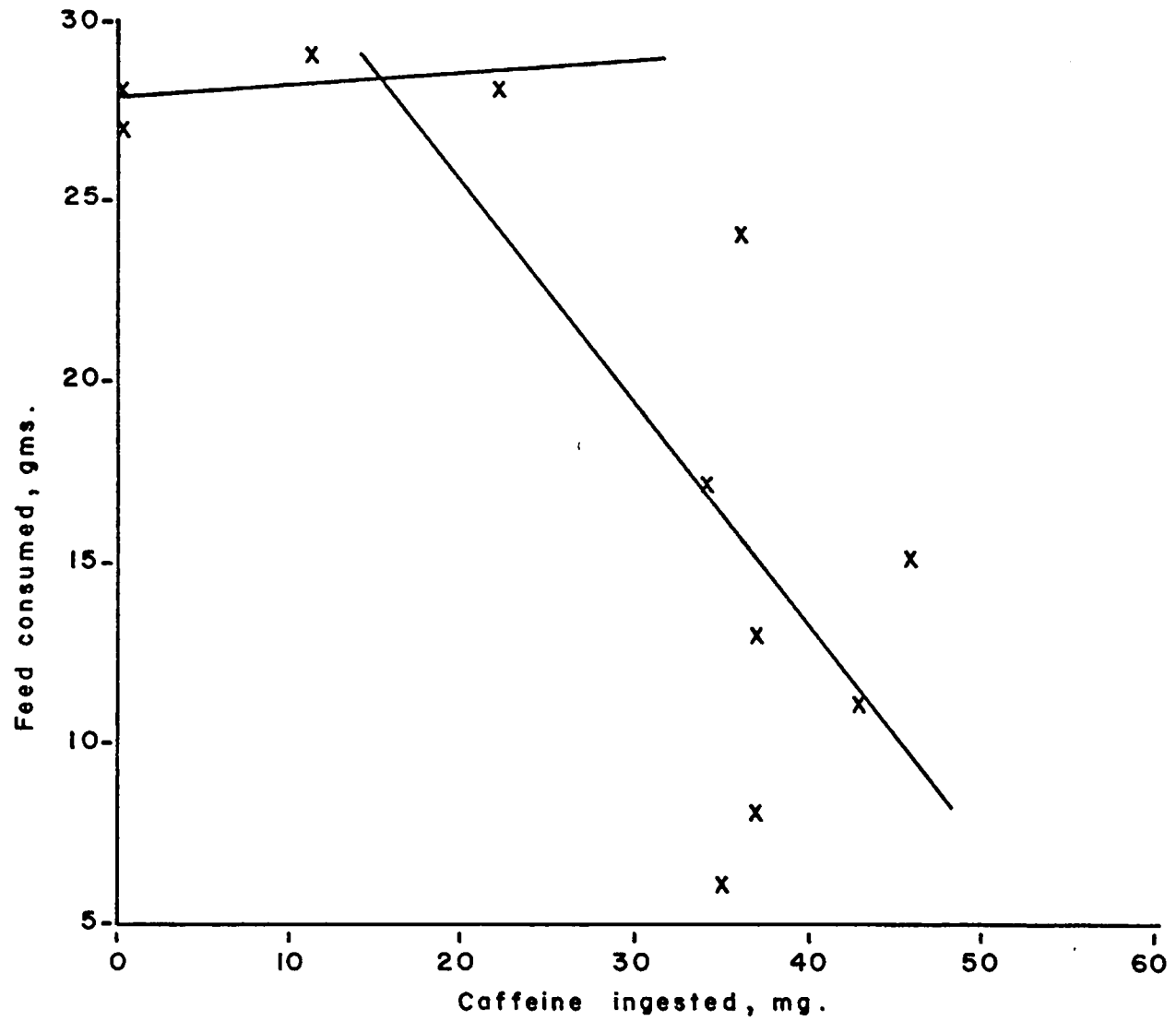


Figure 2. Effect of Caffeine Ingestion on Feed Consumption of Mice

Table 6. Effect of Caffeine on Dietary Nutrient Absorption (M-2)

| Dietary Treatments (ppm added) | % Calcium Absorption | % Phosphorus Absorption | % Nitrogen Absorption | % Fat Absorption |
|--------------------------------------|----------------------------|-------------------------------|-----------------------------|------------------------|
| 0 | 21.05 | 33.13 | 84.98 | 97.43 |
| 2000 | 31.24 | 28.62 | 84.80 | 94.93 |
| 3000 | 22.71 | 24.22 | 83.57 | 95.27 |
| 4000 | 19.94 | 24.46 | 84.04 | 95.75 |
| 5000 | 17.54 | 23.32 | 82.67 | 93.98 |
| 6000 | 23.41 | 25.35 | 82.58 | 95.66 |

The difference in phosphorus absorption found between the control groups for both experiments may be explained by the percentage of available phosphorus of the two diets. The first diet consisted of .342%, assayed (.346% calculated) whereas .509% phosphorus, assayed (.542% calculated) was available in the second experimental diet. Therefore, phosphorus absorption at 54.75% was higher in the controls for the first experiment due to increased digestive efficiency for that nutrient.

The analysis of liver tissue for xanthine oxidase activity showed no statistical differences among the treatments (Table 7). Glutamic oxalacetic transaminase activity of liver homogenates was not significantly altered by 2000 to 6000 ppm caffeine, although an increase was observed compared with the activity of the control group. A significant elevation in glutamic oxalacetic transaminase activity occurred in kidney homogenates of mice fed 6000 ppm caffeine.

The various levels of caffeine fed to mice apparently had no effect on the oxidation of hypoxanthine and xanthine as evidenced by the lack of differences in liver xanthine oxidase activity. It is implied that uric acid formation was not altered by dietary caffeine. In human experiments, Cornish and Christman (1957) found no change in urinary excretion of either xanthine or uric acid after oral administration of caffeine.

Data of liver glutamic oxalacetic transaminase response to caffeine indicates a significantly increased enzymatic activity from 770 to 3030 ppm in the first experiment, although experiment 2 levels

Table 7. Tissue Enzymatic Activity¹ in Response to Dietary Caffeine in Mice (M-2)

| Dietary Treatments (ppm added) | Liver Xanthine Oxidase | Liver Glutamic Oxalacetic Transaminase | Kidney Glutamic Oxalacetic Transaminase |
|--------------------------------|---------------------------------|--|---|
| 0 | 13.23 ^a ² | 80.36 ^a | 61.74 ^a |
| 2000 | 8.47 ^a | 90.05 ^a | 57.06 ^a |
| 3000 | 9.32 ^a | 109.57 ^a | 64.10 ^a |
| 4000 | 13.55 ^a | 105.94 ^a | 67.36 ^a |
| 5000 | 12.30 ^a | 106.26 ^a | 67.61 ^a |
| 6000 | 8.74 ^a | 89.37 ^a | 80.03 ^b |

1. Enzymatic activity is expressed as change in O.D./min./gm. protein. Protein is measured at 280 m μ .
2. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

were not statistically elevated. Analysis of variance of the second experiment showed this lack of treatment differences was due to a high within treatment (error) mean square, denoted by s^2 , and therefore a large standard error of a treatment means ($s_{\bar{x}}$) for the determination of the multiple range test. Differences between treatment means could not be found significant when compared with the least significant ranges (significant studentized ranges, tabular values, times $s_{\bar{x}}$).

Summary

Body weights of mice in experiment 1 were significantly depressed by dietary levels of 1500 and 3030 ppm caffeine. The second experiment showed progressive growth reductions at 2000, 4000, and 6000 ppm caffeine. There was a 20% reduction in the growth rate of mice fed 1500 ppm caffeine, a 30% reduction at 2000 ppm, 46% reduction at 3030 ppm, 54% reduction at 4000 ppm, and 66% reduction at 6000 ppm. The toxic level of caffeine is estimated to be between 770 and 1500 ppm, based on growth. This is similar to the report by Naismith et al. (1969) that weight reductions were found in rats fed 1400 ppm caffeine.

A level of 4000 ppm caffeine produced 8% mortality, 5000 and 6000 ppm produced 17% mortality, respectively, and a 58% mortality rate occurred at 6070 ppm. The feeding of either 12,100 or 24,200 ppm caffeine resulted in 100% mortality. No deaths occurred at levels below 4000 ppm.

Significant reductions in feed consumption generally paralleled growth depressions. Caffeine consumption showed a plateau of intake at dietary levels of 1500 to 6000 ppm.

Caffeine supplementation had no effect on calcium, protein, or fat digestion. Phosphorus utilization may be impaired at 2000 to 6070 ppm as absorptions were decreased in mice fed these dietary levels.

There were no changes in liver enzymatic activity for xanthine oxidase as a result of caffeine treatments of 2000 to 6000 ppm. Liver glutamic oxalacetic transaminase activity was significantly increased at levels of 770 to 3030 ppm in the first experiment; however, an increased response found in the second experiment could not be proven statistically different. Glutamic oxalacetic transaminase activity in kidney tissue was significantly elevated at 6000 and 6070 ppm caffeine. This may suggest possible tissue damage to mice fed 6000 ppm or greater amounts of caffeine.

Upon examination of the parameters measured in these experiments, it appears that the reduced growth of the experimental animals was due to a decrease in appetite and feed consumption, and impairment of phosphorus digestion. An alteration in glutamic oxalacetic transaminase activity may have been a contributing factor at the cellular level.

CHAPTER 3

DIETARY CAFFEINE STUDIES WITH CHICKS

Experimental Procedure

Two experiments were conducted with day old Hubbard broiler chicks from the University of Arizona stock. The birds were randomly distributed among three replicate groups (pens) per treatment and were housed in electrically heated batteries with raised wire floors. Each replicate contained 6 birds (an equal number of males and females). Feed and water were supplied ad libitum for the four week experimental periods.

Caffeine was supplied in increments to the basal diet (Table 8), formulating eight treatments of 0, 55, 111, 222, 444, 888, 1775, and 3550 ppm caffeine for the first experiment and 0, 500, 1000, 1500, 2000, 2500, 3000, and 3500 ppm caffeine for the second experiment. Chromium oxide was included in both experimental diets for the calculation of retentions.

Initial, third and fourth week feed weights were recorded for each pen and third and fourth week body weights were noted. Fecal samples were collected during the fourth week.

Ten animals per treatment were sacrificed upon completion of the second experiment. Livers were removed, frozen immediately with dry ice and stored at -4°C . Tissues were subsequently assayed for glutamic

Table 8. Basal Diet Composition for Both Chick Experiments

| Ingredient | Percent of Diet |
|--------------------------------|--------------------|
| Ground milo | 46.17 |
| Soybean meal | 23.32 |
| Fish meal | 17.12 |
| Whey | 1.00 |
| Dist. dried sol. | 1.00 |
| Animal fat | 5.00 |
| DL-methionine | 0.10 |
| Pr-9 vitamin mix ¹ | 2.50 |
| Dicalcium phosphate | 1.10 |
| Calcium carbonate | 0.75 |
| Salt | 0.20 |
| Trace mineral mix ² | 0.20 |
| Chromium oxide | 0.20 |
| Bentonite | <u>11.34</u> |
| Total | 100.00 |

1. Supplied the following per kg. of diet: 9,925 I.U. vitamin A palmitate, 1,537 I.C.U. vitamin D₃, 5.5 I.U. d-alpha-tocopheryl acetate, 13 mcg. vitamin B₁₂, 2.2 mg. 2-methyl-napthoquinone, 4.5 mg. riboflavin, 28 mg. niacin, 11.3 mg. d-calcium pantothenate, 930.1 mg. choline chloride and 125 mg. ethoxyquin.
2. Supplied as mg./kg. (ppm) of diet: iron 20, FeSO₄·7H₂O; zinc 60, ZnO; molybdenum 1, Na₂MoO₄·2H₂O; manganese 60, MnO₂; calcium 168, CaCO₃; copper 4, CuSO₄·5H₂O; iodine 1.5, KI; and cobalt 1.5, CoCl₂·6H₂O in glucose monohydrate carrier.

oxalacetic transaminase activity, using a Coleman spectrophotometer for recording optical density changes.

Analyses of feed and feces were performed for Cr_2O_3 (Czarnocki et al. 1961), calcium by flame photometry (Coleman 1958), phosphorus (Koenig and Johnson 1942), protein by the Kjeldahl method, and fat. Statistical analyses of data were determined by analysis of variance and Cundan's multiple range test (Duncan 1955).

Results and Discussion

Fourth week body weights of chicks were progressively reduced at 888, 1775, and 3550 ppm caffeine, as indicated by the results of the first experiment in Table 9. There were no statistical differences in the growth of chicks fed up to 444 ppm. Growth rate depressions were 14% at 888 ppm, 37% at 1775 ppm, and 68% at 3550 ppm, when compared with the weight of the control group.

The influence of dietary caffeine level on growth was investigated by linear regression. The equation of the line: $Y = -.1294X + 650.5671$. Correlation between caffeine ppm and body weight was very high as calculated $r = -.9957$ at the 99% level of significance ($P = .01$). The detrimental effect of high caffeine level is evident, based on the criterion of growth.

There were progressive significant reductions in the feed consumption of chicks on dietary levels of 888, 1775, and 3550 ppm caffeine. Feed intake for the four week period was not altered in chicks fed diets containing up to 444 ppm. There were no significant differences in feed conversions due to the respective treatments up to 1775 ppm

Table 9. Effect of Caffeine on Body Weights and Feed Utilization of Growing Chicks (C-1)

| Dietary Treatments (ppm added) | 4th Week Body Weight (gms.) | Feed Consumed Bird/4 Weeks (gms.) | Feed Conversion gms. Feed/gms. Body Weight | Calculated Consumption of Caffeine Bird/4 Weeks (mg.) |
|--------------------------------|-----------------------------|-----------------------------------|--|---|
| 0 | 634 ^{d1} | 1076 ^d | 1.70 ^a | 0 |
| 55 | 641 ^d | 1079 ^d | 1.65 ^a | 59 ^a |
| 111 | 633 ^d | 1079 ^d | 1.71 ^a | 120 ^{ab} |
| 222 | 622 ^d | 1053 ^d | 1.69 ^a | 234 ^b |
| 444 | 621 ^d | 1035 ^d | 1.67 ^a | 460 ^c |
| 888 | 545 ^c | 925 ^c | 1.70 ^a | 821 ^d |
| 1775 | 402 ^b | 734 ^b | 1.75 ^a | 1303 ^e |
| 3550 | 195 ^a | 395 ^a | 2.03 ^b | 1403 ^e |

1. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

caffeine. Feed conversion was least efficient at 3550 ppm, the highest level of caffeine fed. Consumption of caffeine was essentially increased with dietary increments until the intake appeared to remain at a constant level from 1775 to 3550 ppm. Growth and feed intake showed the greatest decline at this plateau of intake.

The dietary treatments did not affect calcium, phosphorus, or fat retention as shown by the data summarized in Table 10. Nitrogen retention appeared to be reduced at 1775 and 3550 ppm caffeine, indicating decreased utilization of this nutrient as a result of feeding high levels of caffeine.

Treatment means from the second experiment, presented in Table 11, generally showed a progressive statistical depression in the growth of chicks for each increasing level of caffeine, greater than 500 ppm. The feeding of 500 ppm had no effect on body weight. Growth rate was reduced 20% at 1000 ppm, compared with the control group. Further reductions in growth were 30% at 1500 ppm, 43% at 2000 ppm, 53% at 2500 ppm and 66% at 3500 ppm.

The decline in body weight associated with increasing dietary caffeine level showed a high degree of correlation, as $r = -.9799$ ($P = .01$). The linear equation, as determined by regression analysis of body weight on caffeine level: $Y = -.1345X + 660.1208$.

A reduced pattern in feed consumption occurred with increasing levels of caffeine greater than 500 ppm. Feed conversions were not significantly altered by increasing caffeine concentration up to a level of 2000 ppm. Feed utilization was then decreased in efficiency

Table 10. Effect of Caffeine on the Retention of Dietary Nutrients (C-1)

| Dietary Treatments (ppm added) | % Calcium Retention | % Phosphorus Retention | % Nitrogen Retention | % Fat Retention |
|--------------------------------|---------------------|------------------------|----------------------|-----------------|
| 0 | 22.25 | 32.15 | 65.51 | 94.92 |
| 55 | 16.14 | 27.46 | 56.41 | 93.43 |
| 111 | 26.09 | 35.48 | 64.94 | 93.26 |
| 222 | 28.10 | 39.30 | 62.66 | 93.94 |
| 444 | 26.76 | 37.91 | 64.93 | 96.42 |
| 888 | 29.42 | 39.44 | 60.30 | 92.93 |
| 1775 | 25.19 | 34.94 | 51.85 | 93.08 |
| 3550 | 28.05 | 42.02 | 51.61 | 91.80 |

Table 11. Effect of Caffeine on Body Weights and Feed Utilization of Growing Chicks (C-2)

| Dietary Treatments (ppm added) | 4th Week Body Weight (gms.) | Feed Consumed Bird/4 Weeks (gms.) | Feed Conversion gms. Feed/gms. Body Weight | Calculated Consumption of Caffeine Bird/4 Weeks (mg.) |
|--------------------------------|-----------------------------|-----------------------------------|--|---|
| 0 | 637 ^{f1} | 1111 ^e | 1.75 ^{ab} | 0 |
| 500 | 662 ^f | 1146 ^e | 1.68 ^a | 573 ^a |
| 1000 | 510 ^e | 926 ^d | 1.79 ^{ab} | 926 ^b |
| 1500 | 445 ^d | 784 ^c | 1.76 ^{ab} | 1176 ^{bc} |
| 2000 | 364 ^c | 684 ^{bc} | 1.88 ^{ab} | 1367 ^{cd} |
| 2500 | 298 ^b | 603 ^{ab} | 2.01 ^{bc} | 1508 ^{de} |
| 3000 | 268 ^{ab} | 590 ^{ab} | 2.19 ^c | 1769 ^e |
| 3500 | 214 ^a | 469 ^a | 2.19 ^c | 1642 ^{de} |

1. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

at the 2500 to 3500 ppm levels. Caffeine consumption increased with dietary level up to 1500 ppm. Intake seemed to level off gradually from the 1500 to 2000 ppm level to 3500 ppm.

Linear regression analysis of calculated caffeine consumption on dietary level was performed for both chick experiments and is shown in Figure 3. The X and Y points plotted are data from Tables 9 and 11. The first line indicates an increase in caffeine consumption with dietary levels up to 1725 ppm, the intersection point. The correlation for this line is very high, as $r = .9860$ at the .01 probability level and there is a good linear relationship between caffeine intake and dietary level to 1725 ppm. The second line shows a decreased intake rate of caffeine. The correlation between caffeine consumption and ppm is not significant as calculated $r = .5754$ (compared with tabular r of .811 at $P = .05$ with 4 error d.f.). Therefore, dietary levels of 1725 to 3550 ppm apparently had little effect on caffeine consumption.

The decreased rate of caffeine consumption, interpreted as a plateau effect, infers that caffeine controlled feed consumption. The influence of caffeine ingestion on feed consumption was analyzed for both experiments by linear regression, illustrated in Figure 4. There is no linear relationship between caffeine intake and feed consumption, as demonstrated by the correlation coefficient for the first equation, $r = .1333$. However, the intersection of the lines indicates a decline in feed consumption at 585 mg. caffeine as the correlation for the second line is very high at $r = .8974$ (compared with tabular $r = .765$, $P = .01$ with 8 error d.f.).

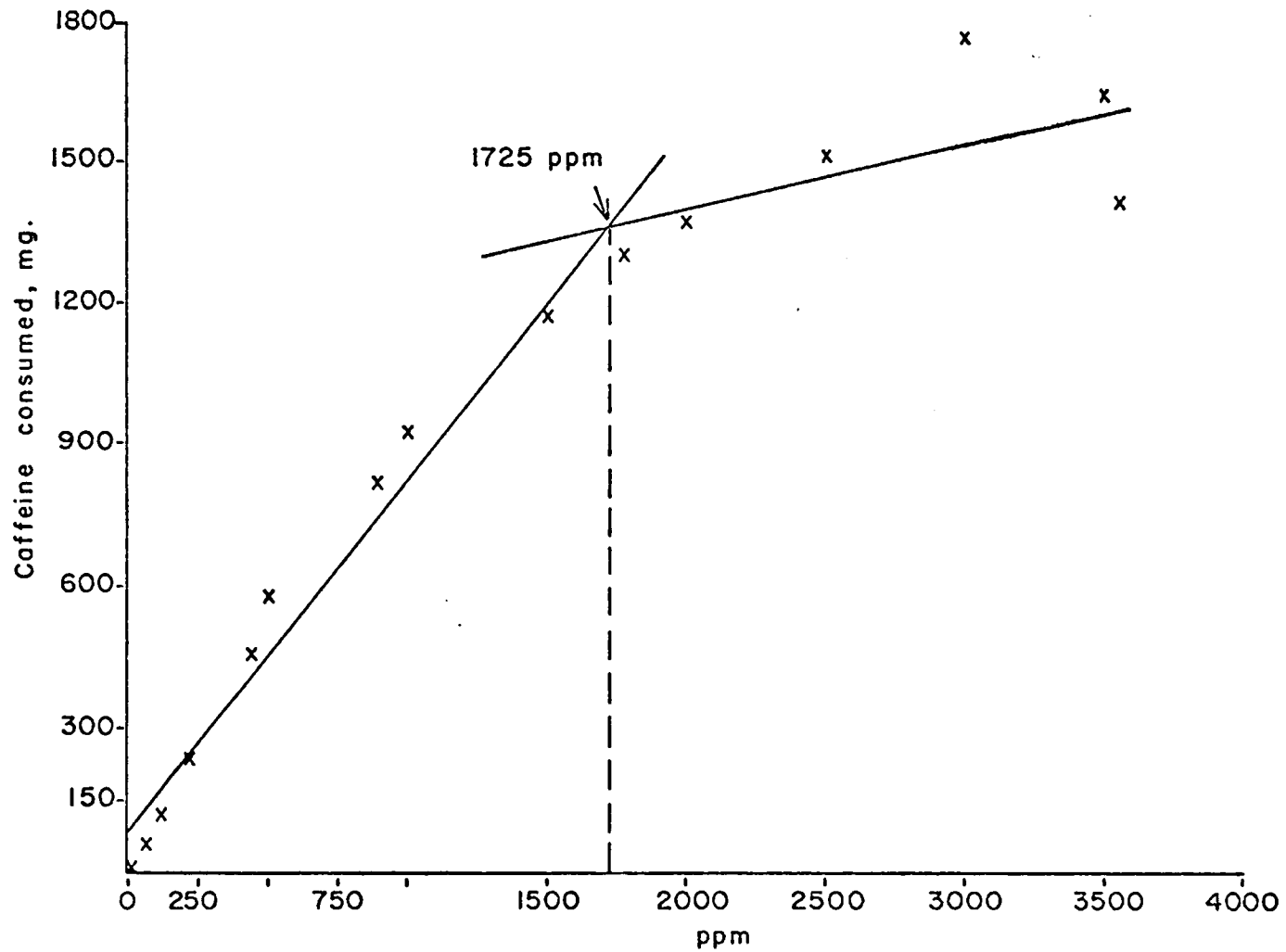


Figure 3. Effect of Dietary Level on Caffeine Consumption of Chicks

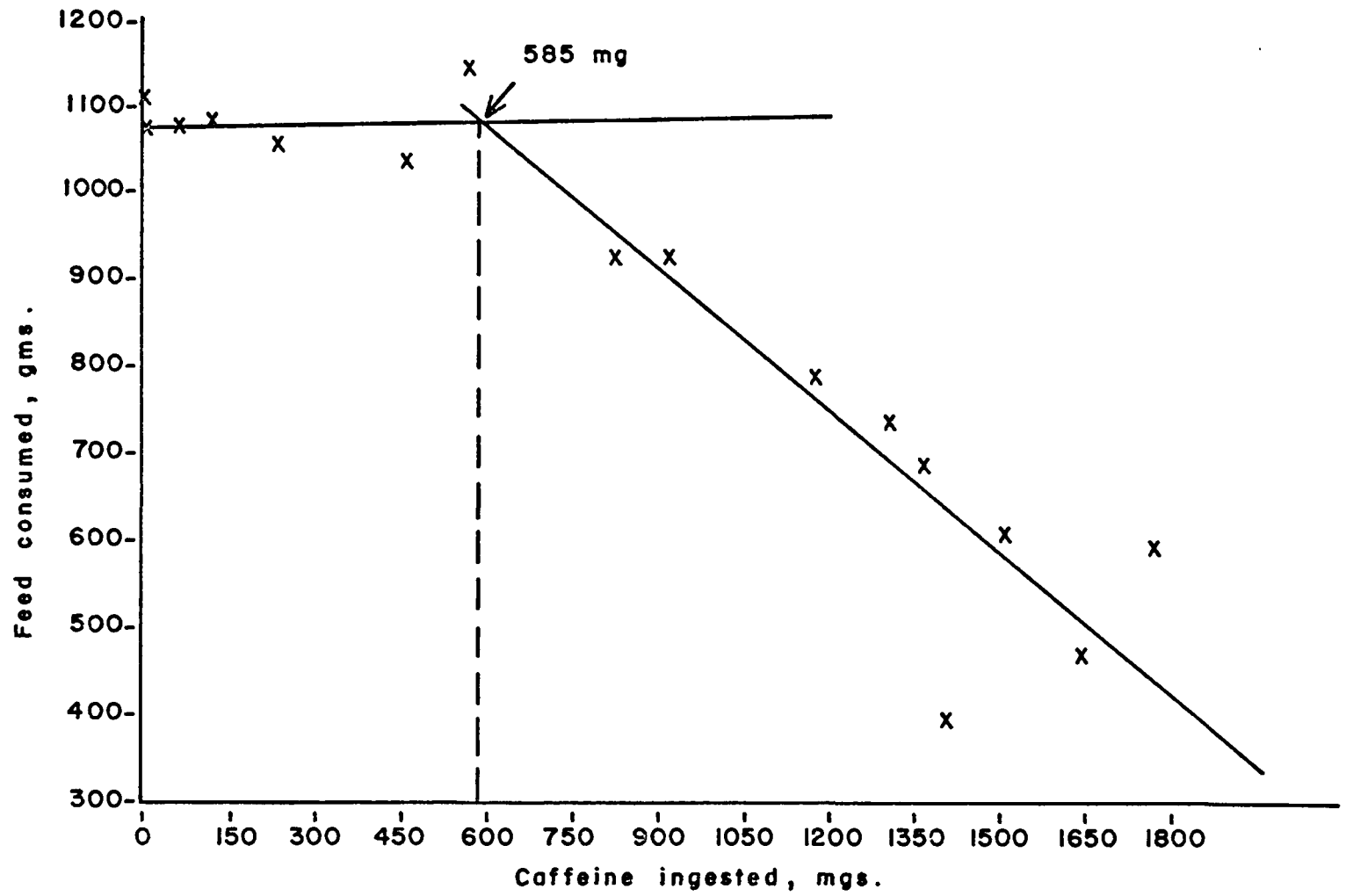


Figure 4. Effect of Caffeine Ingestion on Feed Consumption of Chicks

There were no differences in fat retentions as a result of feeding 500 to 3500 ppm caffeine (Table 12). Treatments of 500 to 2500 ppm had no effect on nitrogen retention whereas 3000 to 3500 ppm both may have depressed nitrogen retention.

Table 13 indicates the mean enzymatic activities for glutamic oxalacetic transaminase in liver tissue, for the various treatments utilized. The statistical difference in the activities found at 1500 and 3500 ppm cannot be interpreted as a true decrease at the highest level. Analysis of variance showed the treatment mean square was less than the error mean square, resulting in a nonsignificant F value of less than 1. Therefore, it is concluded that there were no differences in liver glutamic oxalacetic transaminase activity due to caffeine treatments.

Summary

A level of 888 ppm caffeine or above progressively depressed fourth week body weights of chicks. Toxicity for these animals was apparently between 500 and 888 ppm caffeine. Growth was depressed 14% at 888 ppm, 20% at 1000 ppm, 30% at 1500 ppm, 37% at 1775 ppm, 43% at 2000 ppm, 53% at 2500 ppm, 66% at 3500 ppm, and 68% at 3550 ppm. Chase (1928) reported a marked decrease in the growth of chicks that were controlled fed smaller amounts of caffeine.

Reductions in feed intake showed essentially the same statistical response to dietary caffeine concentration as found with growth depressions. It appears that appetite was adversely affected by a minimum

Table 12. Effect of Caffeine on the Retention of Some Dietary Nutrients (C-2)

| Dietary Treatments (ppm added) | % Nitrogen Retention | % Fat Retention |
|--------------------------------|----------------------|-----------------|
| 0 | 57.73 | 92.29 |
| 500 | 48.45 | 91.31 |
| 1000 | 51.05 | 93.47 |
| 1500 | 51.78 | 91.35 |
| 2000 | 52.08 | 92.00 |
| 2500 | 49.72 | 91.25 |
| 3000 | 36.82 | 89.12 |
| 3500 | 43.61 | 90.68 |

Table 13. Liver Glutamic Oxalacetic Transaminase Activity¹ in Response to Dietary Caffeine in Chicks (C-2)

| Dietary Treatments (ppm added) | Liver |
|-----------------------------------|----------------------------------|
| 0 | 56.05 ^{ab} ² |
| 500 | 57.55 ^{ab} |
| 1000 | 51.40 ^{ab} |
| 1500 | 63.43 ^b |
| 2000 | 53.92 ^{ab} |
| 2500 | 51.75 ^{ab} |
| 3000 | 51.38 ^{ab} |
| 3500 | 43.98 ^a |

1. Enzymatic activity is expressed as change in O.D./min./gm. protein. Protein is measured at 280 m μ .
2. Means not having common letter superscripts are significantly different at the 0.05 level of probability (Duncan 1955).

level between 500 and 800 ppm caffeine. Consumption of caffeine seemed to plateau from approximately 1775 to 3550 ppm. Feed conversions were not statistically altered to 2000 ppm caffeine, indicating utilization of nutrients was equally efficient for the various treatments to this level. The reduction in feed conversions at 2500 to 3550 ppm suggests an inefficiency in nutrient utilization at these high caffeine levels.

Caffeine may have interfered with protein digestion at 3000 to 3550 ppm as evidenced by the reduction in nitrogen retentions at these levels. However, digestion of calcium, phosphorus and fat was not affected by caffeine as retentions of these nutrients were unaltered.

Analysis of liver tissue for activity of glutamic oxalacetic transaminase showed no significant differences among treatments due to dietary caffeine. Clinically, elevation of glutamic oxalacetic transaminase and glutamic pyruvic transaminase activities indicates tissue damage. Therefore, the data showing no change in activity is interpreted as no impairment in the liver function of caffeine-treated chicks.

It is postulated that the reduced growth rates of the chicks resulted from decreased feed consumption, and both inefficient feed utilization and impairment in protein digestion found at the higher levels of caffeine.

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