ACETYL-SALICYLIC ACID IN AVIAN NUTRITION AND METABOLISM

by

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I hereby recommend that this dissertation prepared under my direction by JOHN M. THOMAS

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (CONTINUED)

ABSTRACT

ACETYL-SALICYLIC ACID IN AVIAN NUTRITION AND METABOLISM

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White Leghorn hens were employed to evaluate the effects of feeding acetyl-salicylic acid. Production data for three experiments (10 months each) revealed significant improvements in egg production and feed utilization when dietary levels of 0.05% were fed. These responses were similar in magnitude to that obtained with feeding 25 gms./ton of oxytetracycline. A combination of the two drugs failed to further increase production in two experiments, while in a third study production rate was not different from that obtained with control hens. In a fourth study, five dietary levels (in doubling increments) of from 0.05 to 0.80% acetyl-salicylic acid (ASA) were employed. Hens fed the first three increments attained significantly higher production rates than were exhibited by birds fed the control feed. The two higher levels allowed production rates equal to and significantly depressed from control hens respectively.

Various other parameters measured for all experiments were variable. However, birds fed 0.80% ASA exhibited depressed shell quality, feed intake and body weight.

Six experiments were conducted with New Hampshire x Delaware chicks to evaluate the effects of ASA and some chemical analogs on growth and feed utilization. Data obtained revealed no increased growth from feeding ASA, methylene-di-salicylic acid (MDS) or para-amino-salicylic acid (PASA). A significant increase in growth was noted when acetyl-para-amino phenol (APAP) was fed.

Biochemical evaluations were initiated to attempt an explanation for the observed effects on laying hens. Chicks were also employed for several measurements to extend and expand certain portions of the results obtained with hens. Since this work was of a survey nature, replications were at times sacrificed in order to allow a wider range of biochemical tests.

Visceral fat and adrenal weights were depressed when 0.80% ASA was fed to hens. However, these depressions were not independent of current bird weight. Similar measurements with chicks showed no differences in organ weights which were attributable to treatment. Fatty acid analysis of abdominal and liver lipids revealed no effects attributable to treatments. Plasma protein, creatinine, uric acid and ascorbic acid were numerically decreased while citric acid was numerically increased by the various increments of drug fed to hens.

Similar results were obtained for uric acid when all drugs were fed to chicks. Liver glycogen stores were significantly depressed by the two high levels of ASA while this carbohydrate was increased by all drugs when fed to chicks. Only liver isocitric dehydrogenase was affected (positively) by dietary ASA fed to hens. Five other systems were not affected. Blood plasma levels of salicylic acid were increased in a stepwise fashion when compared to dietary source levels with both chicks and hens. Other drugs fed to chicks did not allow expected plasma values, due principally to specificity of the chemical procedure and the failure of MDS to be hydrolyzed during absorption. Various organs examined (kidney, liver, brain) contained salicylic acid with unexpectedly large amounts found in bile. Bile from chicks fed MDS also contained appreciable salicylic acid indicating that this compound was hydrolyzed after being removed from the blood. Microbial counts on isolates from cecal contents of hens indicated no <u>in vivo</u> antimicrobial effect for ASA.

CHAPTER I

INTRODUCTION

One of the major problems of animal production in many sections of the world is associated with decreased performance during periods of heat-induced stress. This problem is of particular importance with avian species and often results in poor growth and/or reproductive performance. Since a large portion of the year in Arizona is associated with high environmental temperature, this affords an excellent opportunity to study heat-induced stress and associated problems.

The outward manifestations of high environmental temperatures have received considerable attention particularly with laying hens. Decreased rates of egg production during summer months have been reported by Wilham (1931), Hutt (1938) and Hinds (1949). Decreases in egg weight have been reported to be associated with high temperatures by Bruckner (1936), Bennion and Warren (1933), Skogland, Tomhave and Mumford (1951), and Hutchinson (1953). In addition, decreased shell thickness (Warren and Schnepel, 1940) and lowered feed consumption (Wilson, 1949; Lee et al., 1945) have been reported. Mueller (1961) showed a reduction in egg production, feed intake, egg weight and shell quality when comparing a constant temperature of 90°F. as opposed to 50°F.

Kurnick et al. (1964), Huston and Edwards (1961), reported poorer gains and feed utilization with chicks of two different breeds in naturally occurring hot environments. Recently, Milligan and Winn (1964) have shown detrimental effects of elevated controlled temperatures on gain, feed conversion, feathering and pigmentation which were markedly more adversely affected when relative humidities were increased to high levels.

Numerous papers have indicated that elevated body temperatures are exhibited by hens kept in warm environments. Heywang (1938), Lee et al. (1945), and Wilson (1948) have shown increases in rectal temperatures of 0.5-1.5°F. at environmental temperatures of 90°F. or above. Relative humidity was shown by Yeates, Lee and Hines (1941) to be without effect on body temperature of hens maintained at 85°F. with 55% relative humidity while marked increases were encountered at environmental temperatures over 105°F. at a relative humidity of 75%.

That the foregoing outward manifestations are elicited via a stress, namely heat, has been shown by Hill (1961a, 1961b) and Hill, Warren and Garren (1961). Their criteria included some of those originally proposed by Seyle (1936) as part of a "General Adaptation Syndrome" resulting from systemic stress. Some of the general body regulatory hormones are affected by any stressing agent. Most stressors stimulate the anterior pituitary to release higher amounts of adrenocorticotropic hormone (ACTH). Reviews by Bierich, Schonberg and Eckler (1962) and Schindler (1962) indicate that the

mechanism is via a neurohormone termed "corticotropin-releasing-factor" which stimulates the pituitary to produce increased amounts of ACTH. The net result of this phenomenon is increased adrenal weight and increased secretions of glucocorticoids. These steroids are thought to induce any catabolic processes observed during stress.

According to Brown (1959), there is a paucity of knowledge concerning measurements applicable to evaluating stress in poultry. Some of the available measurable parameters of stress in poultry are pituitary enlargement (Garren and Barber, 1955; Garren and Shaffner, 1956), adrenal hypertrophy (Siegel, 1959; Brown, 1959), atrophy of the spleen (Garren and Shaffner, 1956), increase in blood citric acid (Hill, 1961a), and retarded growth, loss of weight and poor reproductive performance previously discussed.

Very few dietary methods for alleviating heat stress have proven successful. Antibiotics have improved the performance of hens during hot weather or artificially-induced high temperatures (Heywang, 1956, 1957a, 1957b, 1959; Assem and Sanford, 1956). Several tranquilizing drugs have been employed to prevent the increased output of ACTH caused by stressors. At low dosage levels various tranquilizers either promoted growth of young chickens (Burger, Van Matre and Lorenz, 1959; Fritz, Wharton and Classen, 1959) or had no effect. Higher levels depressed growth and food intake. Effects of tranquilizers on laying hens have been variable (Hoff et al., 1961; Gilbreath et al., 1960).

Because of the aforementioned high ambient temperatures in Arizona during a large portion of the year, heat stress and concomitant problems are often encountered. No adequate means exist at this time to control these phenomena and the observable parameters of poor growth and production. Since body temperature has been shown to be elevated during periods of high environmental temperatures, it was decided to evaluate acetyl-salicylic acid (aspirin) as an aid in modifying heat-induced stress. The antipyretic and analgesic activities of the salicylates have been well known for many years, but they have never been employed with the avian species. The effect of these drugs on performance, mode of activity and metabolic rate are the general aims of this work.

CHAPTER II

REVIEW OF LITERATURE

Introduction. Some 2400 years ago Hippocrates prescribed the leaves of the willow tree as an aid for the pain of childbirth. The active principle of this and other preparations from the willow was not elucidated until 1826, when Brugnatelli and Fontana announced salicin as the primary medicinal component (Sharp, 1915). From these beginnings salicylate chemistry emerged.

Acetyl-salicylic acid, universally known as aspirin, evolved as a compound of great usefulness when it was found to be non-irritating to gastric mucosa as was not the case for the parent compound, salicylic acid. Apart from its analgesic and antipyretic properties, this compound has been used extensively in the treatment of rheumatic disorders and arthritis. The mechanism of action of the salicylate compounds is still obscure despite a voluminous literature on its medicinal effects.

The early papers dealing with the salicylates have been reviewed by Hanzlik (1927) and a comprehensive work was compiled by Gross and Greenberg (1948). It is the purpose of this review to discuss the salient points of the more recent papers.

Biochemical Effects. Cochran (1952) showed conclusively that plasma salicylate levels from 18 to 50 mg./100 ml. markedly increased oxygen consumption in normal human subjects. Analogous results have been reported by Tenny and Miller (1955) in the dog. Brody (1956) found that slices from several rat organs (excluding kidney) increased in oxygen consumption when animals were given sodium salicylate intraperitoneally (600 mg./kg. body weight). Sproull (1954) confirmed and extended this observation with mouse liver and rat brain slices. Christensen (1959) has observed that in vitro levels of salicylate of 10 to 50 mg./100 ml. caused a release of thyroxine from its combination with plasma proteins. He suggested that increased oxygen consumption in man may be explicable via this mechanism. However, this does not explain the stimulatory effect of salicylate on isolated tissues.

The review by Gross and Greenberg (1948) suggested that the effects of salicylate on protein metabolism, especially non-protein excretory products, were too variable to justify conclusions. Recently, Price and Ford (1963) found that 40 grains of salicylate per day, administered orally to humans, decreased serum uric acid by 1.1 mg./100 ml., while 20 grains per day resulted in an increase of 1.4 mg./100 ml. These results were not subjected to statistical procedures.

The current status of the influence of salicylates on fat metabolism is scanty. Work by Segar and Holliday (1958) indicated development of ketosis and ketonuria in children who received overdoses

of salicylates. Smith (1959) has shown a reduction in liver lipogenesis in rats when plasma salicylate levels of 70 mg./100 ml. occur. Workers in Italy (Bizzi, Codegoni and Garattini, 1964) have given impetus to the notion that salicylates may inhibit the release of free fatty acids without appreciable affect on blood glucose. Para-amino-salicylic acid and salicylamide were ineffective in this respect.

In normal animals one observable effect of salicylate on carbohydrate metabolism is hyperglycemia. Barbous and Herrman (1921) found elevated sugar levels in rabbits, while Sproull (1954) showed the same effect in female mice. A rapid and severe depletion of liver glycogen has been reported in the rabbit (Jackson, 1948), rat (Lutwak-Mann, 1942) and the mouse (Sproull, 1954). This effect may be partially due to adrenal stimulation and in part due to diminished glycogen synthesis since Edelmann, Bogner and Steele (1954) have shown decreased deposition of this compound when fasted rats were fed salicylate and glucose. The situation with respect to muscle glycogen is not resolved with conflicting reports from several workers.

Salicylates have been observed to exert an effect on several enzyme systems. Experiments by Bonstrom and Mansson (1955) showed that sodium salicylate inhibited sulfate exchange with chondroitin sulfuric acid in cartilage; while Cooper, Doty and Almage (1964) found that collagen synthesis was inhibited by salicylates. Kaplan, Kennedy and Davis (1954) concluded that 2-oxyglutarate dehydrogenase

and succinic dehydrogenase were markedly inhibited by salicylate *in vitro*. The enzyme, xanthine oxidase, was inhibited during *in vitro* experiments with rat tissues by Lutwak-Mann (1942). Mitidieri and Affonso (1959) reported plasma salicylate levels of 30 mg./100 ml. caused depressed activity of this enzyme in rat livers. Manso, Taranta and Nydick (1956) observed elevated serum transaminase levels in more than 50% of children receiving aspirin. Other workers (Penniall, Kalnitsky and Routh, 1956) have shown that serum levels of 137 mg./100 ml. of salicylate have no effect on hexokinase, cytochrome oxidase, DPNH-oxidase or DPNH-cytochrome c reductase. Lower concentrations have been shown to inhibit 2-oxyglutarate dehydrogenase and succinic dehydrogenase (Kaplan, Kennedy and Davies, 1954).

Several workers (Brody, 1956; Penniall, 1958) have indicated that salicylates should be considered among agents which uncouple oxidative phosphorylation. Among the changes which might be expected to occur with an uncoupling of oxidation and phosphorylating reactions are decreased ATP and creatine phosphate levels along with accumulations of inorganic phosphate without a depression in oxygen consumption. These effects have been observed using isolated rat diaphram incubated with salicylate (Smith and Jeffrey, 1956).

CHAPTER III

REPRODUCTIVE AND BIOCHEMICAL EFFECTS OF ACETYL-SALICYLIC ACID FED TO LAYING HENS

Experimental. Four experiments were conducted during three different years to evaluate the effect of acetyl-salicylic acid on reproductive performance of laying hens. Experiments 1 and 2 utilized Single-Comb White Leghorns from the University of Arizona strain, while Experiments 3 and 4 were conducted with a commercial strain, White Leghorn. A diversity of environments was sampled in that the first two experiments were conducted in floor pens while the third was carried out in open range shelters. The fourth experiment employed birds housed in individual laying cages in a conventional open cage house. Birds were randomly distributed into four replicate groups of 20 and 25 hens/pen in Experiments 1 and 2, while 40 birds/pen and two replicates were employed in Experiment 3. In Experiment 4, four replicate groups of 5 birds each were fed each experimental diet. The first three experiments were conducted for 40 weeks and the fourth for 32 weeks. Egg production (hen - day) and feed consumption data were summarized at 28-day intervals in all studies. Eggs were obtained for hatchability estimates in Experiment 3 and egg quality evaluations (Haugh, 1937a) were made in both

Experiments 3 and 4 at suitable intervals. Birds and feed were weighed at applicable times, and feed and water were supplied ad libitum. The compositions of the basal diets are given in Table 1. In the first three experiments acetyl-salicylic acid (ASA) was fed at a level of 0.05% alone and in combination with oxytetracycline at a level of 25 gms./ton. Increasing levels of ASA were employed in the fourth experiment (Table 5).

The various biochemical measurements reported in this chapter were performed on samples obtained from birds in Experiment 4. Blood plasma samples (heparinized and centrifuged whole blood) were obtained by cardiac puncture, decapitation or venous puncture depending on the amount of sample needed. Various organs were excised from decapitated hens and enzyme activities were determined on fresh homogenates of these tissues. Otherwise, the tissues were immediately frozen (-15°C.) and other determinations accomplished at a more convenient time. Plasma salicylic acid was determined by the method of Brody, Underfriend and Coburn (1944); reduced ascorbic acid using the procedures of Bessey (1938) and Mindlin and Butler (1938); uric acid as given by Brown (1945); and creatinine by the procedure of Folin and Wu (1919), as modified by Peters (1942) and Bonsnes and Taussky (1945). The latter two methods entailed treatment of the plasma to obtain a protein-free filtrate (Folin and Wu, 1919). Serum proteins were estimated from values read from a refractometer (American Optical Company, Los Angeles, California)

Table 1. Experimental Diets for Hens

Ingredients	Experiments 1 & 2 %	Experiments 3 & 4 %
Yellow corn	10.00	6.45
Milo	55.00	56.00
Dehydrated alfalfa meal (17%)	5.00	5.00
Soybean meal (44%)	18.00	14.50
Fish meal (65%)	1.00	-
Meat and bone scraps (50%)	1.00	1.50
Hydrolyzed animal and vegetable fat	1.00	3.00
Cottonseed meal (41%)	-	2.00
Methionine hydroxy analog	-	0.10
Sodium chloride	0.50	0.35
Ground limestone	5.00	6.25
Dicalcium phosphate	1.50	2.25
Manganese sulfate pentahydrate (70%)	0.02	-
Trace mineral mix	-	0.10[b]
Vitamin premix	2.00[a]	2.50[c]
Total	100.02	100.00

[a] Supplied the following per pound of diet: 2000 I.U. vitamin A, 700 I.C.U. vitamin D3, 2.0 gm. riboflavin, 5.0 mg. niacin, 2.5 mg. calcium pantothenate, 227.0 mg. choline chloride and 6.0 gm. soybean meal as a carrier.

[b] Supplied the following in ppm: 20 iron, 60 zinc, 1 molybdenum, 60 manganese, 168 calcium, 4 copper, 1.5 iodine and 1.5 cobalt.

[c] Supplied the following per pound of diet: 4500 I.U. vitamin A, 700 I.C.U. vitamin D3, 2.5 I.U. vitamin E, 6 mcg. vitamin B_{12}, 1.0 mg. vitamin K, 2 mg. riboflavin, 12 mg. niacin, 5 mg. calcium pantothenate, 425 mg. choline chloride, 56.75 mg. ethoxyquin (as a preservative) and 8.4 gms. soybean meal as a carrier.

calibrated to read in gms./100 mls. Glycogen in muscle and liver was determined using a modification of the method of Good (1933). After appropriate hydrolysis, total reducing sugars were estimated by the procedure of Morris (1948). Salicylic acid contents of various organs and tissues were determined as follows: 2 gms. of tissue were homogenized with 45 ml. borate buffer (pH 10), centrifuged, the supernatant decanted into a 250 ml. beaker, 45 ml. ethylene dichloride and 2 ml. 6N HCL were added and the solution stirred 7 minutes. From this point the procedure was as given by Brody, Udenfriend and Coburn (1944). Fatty acid determinations were accomplished by saponification of chloroform extracts of the various samples and subsequent gas-liquid-chromatography of methyl esters prepared by heating with boron-trifluoride in methanol. A five foot column employing 15% diethylene-glycol-succinate on chromosorb W (60/80 mesh) was used.

Determinations of various enzyme activities were carried out on fresh liver homogenates (Krebs-Ringer phosphate solution) using appropriate dilutions to obtain the activities in measurable ranges. At least one liver from each treatment was employed for each daily run. Xanthine dehydrogenase and the Kreb's cycle enzymes were determined as suggested by Umbreit, Burris and Stauffer (1959) using a Precision Warburg Respirometer. Glutamic-oxalacetic transaminase was determined by the method of Karmen (1955), isocitric dehydrogenase as per Sigma (1961), succinic dehydrogenase as given

by Cooperstein, Lazarow and Kurjus (1950), and cytochrome oxidase by the method of Cooperstein and Lazarow (1951). Solutions containing the proper biochemicals were placed in cuvettes and subsequent changes in optical densities read in a Beckman Model B Spectrophotometer at suitable wavelengths. These changes were recorded on a Bausch and Lomb V. O. M-5 Strip Chart Recorder. All results presented are in arbitrarily selected units and are corrected for temperature.

Cecal samples for microbiological counts were collected from hens near the termination of Experiment 4. Thirty-six hens (6/treatment) were removed from the cage laying house and placed in a laying battery. Three weekly samples were collected, each at 5:00 a.m. Samples were aseptically weighed and placed in an applicable volume of sterile 0.1% tryptone solution to produce a 1-100 dilution. Serial decimal dilutions were made to appropriate multiples of 10 for plate counts. Inoculations from such dilutions were made into the suitable sterile media. Total counts were made using tryptone glucose extract, yeast counts were made with potato-dextrose agar acidified to pH of 3.5 with citric acid, coliform organisms were enumerated by plating on eosin-methylene blue agar, lactic acid bacteria were counted on micro assay agar (Difco) and molds were viewed on potato dextrose agar (unacidified). The plates (in duplicate) were incubated at 37°C. for 72 hours before counting. Media were obtained from Difco Laboratories, Detroit, Michigan. After the counts were made, various organisms were transferred from each agar

plate to slants of the same medium. Subsequently, each organism was tested for sensitivity to acetyl-salicylic acid by using various concentrations of this drug pipetted on paper discs in seeded plates.

Appropriate statistical techniques were employed in evaluating the production and laboratory data. Analysis of variance (Snedecor, 1956) was routinely used and mean differences were judged by Duncan's (1955) multiple range test. When unequal replication was encountered, Kramer's (1956) procedure was employed. In combining production data from 28-day periods into total summaries, the individual error mean squares were shown to be from the same population using an approximate procedure found in Table 31 of Pearson and Hartley (1954). Borderline values were examined by the procedure of Bartlett (1937). In all analyses of this type no period by treatment effects were noted and only one analysis of period data indicated that it contained an error variance which was from a different population of variances. This data was retained.

Results and Discussion. Egg production was significantly improved in Experiment 1 when acetyl-salicylic acid, oxytetracycline or a combination of these two drugs was fed and performance compared with that obtained using the basal diet (Table 2). Production values represent the entire 40-week study. The combination of drugs failed to produce a significant response over that obtained when either compound was fed alone. No significant effects on feed conversion were obtained.

Table 2. Effect of Acetyl-Salicylic Acid and Oxytetracycline on the Performance of Laying Hens

Supplement to basal diet	Egg prod. %	Feed/ doz. eggs lbs.	Average feed consumption per day gms.
None	56.2a[1]	4.37	96
Acetyl-salicylic acid, 0.05%	60.5b	4.46	101
Oxytetracycline, 25 gms./ton	61.0b	4.31	98
Acetyl-salicylic acid, 0.05%, 25 gm./ton oxytetracycline	61.5b	4.27	100

[1] Means having different superscripts are significantly different at the 0.05 level of probability. Each mean is based on four replicate groups of 20 hens each.

In the second experiment the same dietary treatments were employed. The data, presented monthly, (Table 3) illustrate that increased egg production was obtained throughout the year when acetyl-salicylic acid was fed. Since heat stress was encountered in this study (periods 7-10), it was anticipated that a large part of any observable reproductive effect would be evident at this time, especially in lieu of the known antipyretic effects of aspirin. Since this was not the case, the mode of action of this drug must have been due, at least in part, to another property or properties. The overall increases in production were again significant when compared to that allowed by the basal diet. A significant improvement in feed conversion was also obtained with the feeding of oxytetracycline.

Feeding of both compounds failed to produce a response in egg production in Experiment 3 (Table 4). However, both compounds fed alone again elicited significant responses in production when compared to that obtained from hens fed the control diet. The response produced by acetyl-salicylic acid was significantly greater than that found with oxytetracycline fed alone. Both compounds significantly improved feed conversion while all treatments were ineffective with reference to feed intake, egg weight and hatchability of fertile eggs. A significant improvement was noted in interior egg quality when oxytetracycline was fed alone. Perhaps the most interesting response observed was a significant improvement in shell

Table 3. Effect of Acetyl-Salicylic Acid and Oxytetracycline on Monthly Egg Production and Feed Conversion of White Leghorn Pullets

Period No.	Period Dates	Temp. average °F. Max.	Temp. average °F. Min.	Supplement to Basal Diet: None % Prod.	None Feed conv.[1]	0.05% Acetyl-salicylic acid % Prod.	0.05% Acetyl-salicylic acid Feed conv.	Oxytetracycline (25 gm./ton) % Prod.	Oxytetracycline (25 gm./ton) Feed conv.	Acetyl-salicylic acid + oxytetracycline % Prod.	Acetyl-salicylic acid + oxytetracycline Feed conv.
1	12/5 -1/1	64	43	74.2	3.77	80.9	3.72	76.9	3.46	79.1	3.64
2	1/2 -1/29	60	36	67.7	4.55	74.9	4.77	77.3	4.13	72.7	4.17
3	1/30-2/26	71	43	68.5	4.34	76.5	3.74	76.5	3.90	74.0	4.23
4	2/27-3/26	72	44	68.7	4.45	76.7	4.32	72.8	3.99	73.7	4.18
5	3/27-4/23	78	49	67.3	4.29	73.4	4.17	71.1	3.85	69.5	4.23
6	4/24-5/21	89	60	65.4	3.79	67.6	3.83	69.4	3.67	66.6	3.80
7	5/22-6/18	94	63	59.0	3.84	63.6	3.72	66.9	3.54	64.0	3.72
8	6/19-7/16	97	75	50.1	3.97	54.5	3.93	52.7	3.59	55.3	3.73
9	7/17-8/13	90	72	41.3	4.95	48.5	4.86	48.0	4.42	46.4	4.67
10	8/14-9/10	92	71	44.5	4.94	50.5	4.76	50.7	4.43	52.0	4.65
	Average			60.7^{a1}	4.29^{a}	66.7^{b}	4.15^{a}	66.2^{b}	3.90^{b}	65.3^{b}	4.10^{ab}

[1] Averages having different superscripts are statistically different at the 0.05 level of probability. Each mean is based on four replicate groups of 25 hens each.

Table 4. Effect of Acetyl-Salicylic Acid and Oxytetracycline on Reproductive Performance, Egg Quality and Hatchability

Supplement to basal diet	Egg prod. %	Feed/dz. eggs lbs.	Avg. feed cons./dz. gms.	Avg. egg wt. gms.	Shell percent %	Haugh units	Hatch-ability %
None	64.98[a][1]	4.69[b]	114[a]	58.4[a]	9.16[a]	75.11[ab]	78.1[a]
Acetyl-salicylic acid, 0.05%	71.11[c]	4.24[a]	114[a]	58.2[a]	9.34[b]	75.37[b]	82.0[a]
Oxytetracycline, 25 gms./ton	68.82[b]	4.36[a]	113[a]	58.4[a]	9.33[b]	73.72[a]	80.8[a]
Acetyl-salicylic acid 0.05%, 25 gms./ton oxytetracycline	65.41[a]	4.64[b]	112[a]	58.4[a]	9.34[b]	75.83[b]	84.6[a]

[1] Means having different superscripts are significantly different at the 0.05 level of probability. Each mean is based on two replicate groups of 40 birds each.

quality allowed by all dietary treatments. For the sake of brevity, the monthly results are not presented since these data followed the same general pattern as in Experiment 2 and no period-by-treatment effects were observed. This would substantiate the statement made in the discussion of Experiment 2 that the action of acetyl-salicylic acid is, at least in part, independent of its antipyretic properties.

In the first two experiments using University strain Leghorns in an enclosed house, the combination of drugs failed to allow a response of greater magnitude than either drug fed alone. The production level supported was significantly superior to that of the control hens in both studies. However, in the third experiment, employing commercial birds in range shelters, the production level supported by the combination of drugs was not different from that found for control birds. No satisfactory explanation can be offered for this apparent discrepancy unless the failure was involved with location and strain.

Since it was thought that three experiments during three years was sufficient to indicate the efficacy of acetyl-salicylic acid in stimulating egg production, a fourth experiment was designed to investigate the possible toxic level of this drug. The results, dealing with production and other traits for the cooler and warmer portions of the year, are given in Table 5 together with a summary for the complete year.

Table 5. Effect of Levels of Acetyl-Salicylic Acid on Egg Production, Feed Efficiency, Egg Quality and Egg Weight of White Leghorn Pullets

Level of dietary acetyl-salicylic acid (%)	Percent production[1]			Feed/dz. eggs (lbs.)			Gain or loss in wt. (gms.)	Avg. feed cons./ day (gms.)		
	C[2]	W	T	C	W	T		C	W	T
None	74.51^{b}	56.79^{a}	64.67^{b}	4.12^{bc}	4.87^{c}	4.53^{c}	+181	115^{bc}	96^{b}	104^{b}
0.05	81.29^{cd}	69.06^{b}	74.50^{d}	3.90^{ab}	3.99^{ab}	3.95^{ab}	+ 56	119^{c}	102^{b}	110^{c}
0.10	76.56^{bc}	65.43^{b}	70.38^{cd}	3.86^{ab}	3.97^{ab}	3.92^{ab}	+150	112^{b}	97^{b}	104^{b}
0.20	82.32^{d}	70.34^{b}	75.67^{d}	3.66^{a}	3.83^{a}	3.75^{a}	+ 93	113^{b}	100^{b}	106^{bc}
0.40	76.02^{b}	58.99^{ab}	66.56^{bc}	3.84^{ab}	4.61^{bc}	4.27^{bc}	+209	110^{b}	101^{b}	105^{bc}
0.80	59.07^{a}	51.37^{a}	54.79^{a}	4.35^{c}	4.46^{abc}	4.41^{c}	-176	96^{a}	82^{a}	88^{a}

[1] Averages having different superscripts are statistically different at the 0.05 level of probability. Each mean is based on four replicates of 5 birds each.

[2] C = cool weather, January 2 - April 23, mean high temperature = 71.30°F.
W = warm weather, April 24 - September 8, mean high temperature = 88.40°F.
T = total experiment, January 2 - September 8, mean high temperature = 80.78°F.

Table 5. Continued

Level of dietary acetyl-salicylic acid (%)	Avg. egg wt. (gms.)			Haugh units			Shell percentage		
	C	W	T	C	W	T	C	W	T
None	59.18^{c}	59.85^{c}	59.55^{e}	71.96^{a}	68.81^{a}	70.35^{a}	9.35^{c}	9.05^{c}	9.19^{c}
0.05	58.21^{bc}	59.67^{c}	59.02^{de}	75.40^{ab}	71.65^{ab}	73.47^{a}	9.09^{bc}	8.80^{bc}	8.91^{bc}
0.10	56.93^{a}	58.77^{bc}	57.95^{bc}	74.71^{ab}	70.73^{a}	72.71^{a}	9.21^{bc}	8.82^{bc}	9.00^{bc}
0.20	57.65^{ab}	59.23^{c}	58.53^{cd}	73.33^{ab}	70.44^{a}	71.88^{a}	9.03^{bc}	8.56^{b}	8.70^{ab}
0.40	56.39^{a}	56.54^{a}	56.48^{a}	72.86^{a}	70.26^{a}	71.50^{a}	8.89^{ab}	8.42^{ab}	8.65^{ab}
0.80	56.41^{a}	57.66^{ab}	57.10^{ab}	78.16^{c}	75.73^{b}	76.85^{b}	8.62^{a}	8.15^{a}	8.39^{a}

Egg production was significantly improved with the first three levels of acetyl-salicylic acid for both periods and the total yearly results. The fourth level (0.40%) allowed egg production equal to that attained with the control birds while the highest level elicited significantly depressed production in the cooler months and, consequently, the total production for the year. During the warmer portion of this study, the depression was not significant. This may have been due to depressed feed intake during this period which did not allow as much of the drug to be ingested. Feed conversions were similarly affected although the effect on this criterion was not as readily apparent. Regression analysis of both factors for the year did not reveal any effects attributable to a linear response while the magnitude of the residual term suggested some type of non-linear fit.

Haugh units for the year were unaffected by dietary treatment except for birds fed the highest level of ASA. The significantly increased interior egg quality noted with these hens was probably directly related to significantly depressed egg production. Shell percentages were significantly depressed when birds were fed the highest levels of the drug. However, the depression was linear for all levels when regression analysis was applied. Since only the first level of this drug was employed in Experiment 3 (allowing a significant increase in shell percentage), the only applicable comparison in Experiment 4 is with the 0.05% level of the drug. The slight depression noted was not significant. These contradictory

results may indicate that low levels of drug are either beneficial or without effect while higher levels have some property affecting calcium metabolism and, thereby, alter shell percentage. There is only slight evidence in this regard, since birds fed the highest level of the drug also exhibited depressed production. Therefore, it might be expected that their shell percentage would at least be equivalent to that exhibited by the control hens. However, their feed intakes were significantly depressed which could explain some part of the observed depression if an effect on calcium metabolism is also assumed. Feed intake might also be used as a plausible argument for an involvement for ASA in calcium metabolism when the first two groups are compared, i.e., shell percentages vs intakes. However, these birds also exhibited significantly increased production which would account for increased intake and a slight depression in percentage of shell.

The loss in body weight observed when the hens consumed feed containing the highest level of drug can be largely explained by depressed feed intake exhibited by the birds. The question as to whether this depression was due to palatability of the ration or some metabolic effect decreasing appetite remains for future investigation.

The foregoing observed effects on reproduction and other measurable traits were not explainable on the basis of the known properties of ASA. Therefore, further studies were initiated using

birds from the fourth experiment to attempt an explanation of the responses obtained from the feeding of this drug. The toxic effects noted with the highest level of this dietary supplement were also investigated. It seemed that a productive approach for studying the latter phenomena might be an investigation into the observed loss in body weight by these birds. Accordingly, seven birds from each dietary treatment were weighed and sacrificed. Liver, adrenal glands (2) and visceral fat were excised and weighed. Adrenal glands were chosen since stress is known to affect their size. The liver is the site of a large portion of the metabolic activity of the body. Amount of visceral fat was evaluated since it was noted that birds fed the highest level of drug had exhibited poor handling quality during a previous weighing period. The results are presented in Table 6.

Again, as in the overall experiment, a significant depression in body weight occurred. However, these results are not comparable to those in Table 5, since those figures were based on weight change while these under consideration are only for a particular given time and are related to initial body weight. Even though there are obvious differences (some of which were significant) in the organ weights presented, it was found by covariance procedures that these means were almost entirely related to the observed body weights. Means adjusted by covariance are presented in parentheses, adjacent to each observed mean (Table 6). These adjusted means were not

Table 6. Body Weight and Some Organ Weights of Birds Fed Levels of Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Body weight lbs./bird[1]	Visceral fat gms./bird	Adrenal glands mgs./2 adrenals	Liver gms./liver
None	4.36[a]	62.16[ab](50.80)[2]	174.13 (168.9)	44.66 (42.5)
0.05	4.54[a]	85.86[a] (65.01)	183.22 (173.6)	43.60 (39.6)
0.10	4.36[a]	77.39[a] (66.18)	189.80 (184.7)	43.91 (41.8)
0.20	4.04[ab]	59.19[ab](65.28)	176.73 (179.5)	38.00 (39.2)
0.40	4.21[a]	65.70[a] (62.71)	168.94 (167.6)	38.64 (38.1)
0.80	4.32[b]	22.31[b] (62.75)	151.09 (169.6)	34.99 (42.7)

[1] Averages having different superscripts are statistically different at the 0.05 level of probability. Each mean is the average of seven measurements.

[2] Adjusted treatment means. These means are the result of covariance techniques.

significantly different from one another. However, the observations (adjusted) indicate a tendency for increased visceral fat at all levels of drug compared with the unsupplemented control birds. These increases should be judged with caution since both, dependent and independent variables, contain significant effects due to treatment with some inherent dangers in the applicability of the statistical techniques employed (Steel and Torrie, 1960). The adjusted adrenal weights at the lowest three drug levels were also slightly increased. There was a lack of apparent effect on liver weight. Another method of explaining the effects on visceral fat (observed means) would be to reverse the argument and state that one effect of ASA may be in reduction or suppression of fat absorption or metabolism and, thence, an effect on body weight via decreased absorption and/or deposition of fat.

In order to substantiate that the differences in observed body fat were not due to an error in metabolism but rather due to decreased deposition or absorption, it was decided to investigate the fatty acid composition of the visceral fat. The results are presented in Table 7. Any fatty acids which appeared only in trace amounts were not reported.

Individual statistical treatment of these results showed only one difference (not indicated in Table 7) to be significant. This difference was the increased stearic acid content found in fat from birds fed the highest level of ASA. This value was significantly

Table 7. Fatty Acid Composition of Visceral Fat Excised from Hens Fed Levels of Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Myristic[a] C:14[b]	Palmitic C:16	Palmitoleic C:16:1	Stearic C:18	Oleic C:18:1	Linoleic C:18:2
None	1.26	19.8	5.4	7.3	49.8	16.5
0.05	1.11	20.1	4.9	6.4	53.1	14.4
0.10	1.07	19.7	4.4	6.8	52.0	16.0
0.20	1.29	19.6	4.7	7.4	50.9	16.2
0.40	1.20	20.9	5.1	7.2	50.7	14.9
0.80	1.20	19.3	4.5	10.4	50.1	14.5
	0.11[c]	1.03	0.43	0.78	1.82	0.85

[a] Tabled values are in percentage. Each mean is the average of three replicate determinations.

[b] Indicates number of carbon atoms and number of points of unsaturation.

[c] Pooled estimates of the standard error of the mean for each fatty acid.

altered when compared to all other means in the stearic acid column. However, no particular importance is indicated by this finding since the loss from other fatty acids in this line (0.80% ASA) in the table shows no particular contribution at the expense of another fatty acid or acids. These results indicate no metabolic error in fat metabolism, unless it was one of rate limiting deposition. The fatty acid composition of livers and kidneys obtained from the organ weight trial was also subjected to fatty acid composition analyses. Kidneys were obtained from the last two hens sacrificed in each treatment group; while two livers per treatment were selected for analysis. The results of the liver analyses are shown in Table 8. The kidney fatty acid patterns obtained were similar to those obtained for the liver samples.

As would be expected, utilizing less experimental material, the pooled estimates of the mean standard errors were much larger than was the case for visceral fat. These determinations were repeated and similar answers obtained, indicating that the variation was in the organs rather than errors present in the chemical procedures. It appears on examination of the results that stearic acid was somewhat increased by the three higher levels of ASA. However, it is again difficult to ascertain any trends as to which other fatty acids were decreased. Perhaps this could be done in the 0.80% ASA portion implicating particularly palmitic acid. The writer feels that this is a dangerous procedure based on the amount

Table 8. Fatty Acid Composition of Livers Excised from Hens Fed Levels of Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Myristic[a] C:14[b]	Palmitic C:16	Palmitoleic C:16:1	Stearic C:18	Oleic C:18:1	Linoleic C:18:2
None	1.48	28.3	4.3	11.0	45.7	9.4
0.05	1.55	29.2	4.6	10.0	47.6	6.9
0.10	0.69	26.3	4.2	10.6	52.3	5.9
0.20	0.82	30.1	2.5	15.2	45.3	6.1
0.40	0.57	27.0	2.7	15.0	48.2	4.5
0.80	1.90	23.2	2.9	15.7	48.4	7.9
	0.65[c]	2.77	0.83	3.15	4.96	0.90

[a] Tabled values are in percentage. Each mean is the average of two replicate determinations.

[b] Indicates number of carbon atoms and number of points of unsaturation.

[c] Pooled estimates of the standard error of the mean for each fatty acid.

of replication and the variability inherent in these replicates. It may be tentatively concluded that no major shifts in fatty acid patterns were evident in visceral fat, liver or kidney. The lack of body fat in birds fed the highest level of ASA would then be the result of decreased rate of deposition, absorption or retention.

The data in Table 6 also contained some evidence for adrenal weight change both for adjusted and non-adjusted means. If the effects of ASA were associated with decreased stress, it would be expected that adrenal size would decrease. Unadjusted means tend to agree with this hypothesis while adjusted means tend to support the idea that low levels of ASA tend to increase adrenal size, perhaps increasing function, while higher drug levels return them to "normal" size. Two approaches were taken to attempt a more meaningful answer to this portion of the problem. If in fact adrenal weight increased, then accelerated metabolic activity should follow allowing an increased level of blood citric acid. Some evidence, although contradictory reports exist, points to depleted adrenal ascorbic acid during stress. Some increase in plasma ascorbic acid is generally attributed to this effect. The second approach was to investigate both protein and carbohydrate metabolism. Results applicable to the first approach (3 hens per treatment) are found in Table 9.

The results indicate that all levels of ASA fed depressed ascorbic acid, while the highest three levels of drug employed

Table 9. Plasma Citric and Reduced Ascorbic Acids Found in Hens Fed Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid	Ascorbic acid reduced mcg./ml.	Citric acid mcg./ml.
None	10.19[a]	33.63
0.05	7.26	38.40
0.10	6.18	38.67
0.20	8.90	49.26
0.40	5.96	44.19
0.80	9.43	44.61
	0.97[b]	6.57

[a] Each mean is the average of three replicate determinations.

[b] Pooled estimates of the standard error of the mean.

allowed the highest blood plasma citric acid. The control group exhibited levels numerically smaller than all other treatments employed. Biological variation was high (30.0% C.V.) in the citric acid portion which allowed no statistical inference. However, using ascorbic acid error was more controlled, and when treatment sum of squares was partitioned into a component relating all means to the control, a significant ($P < 0.05$) depression was noted. These values would tend to indicate that adrenals in fact decreased in size unadjusted weights, although no reasonably good observable correlation existed between these values and plasma ascorbic acid. Since different birds were employed for each criterion, this type of comparison is precarious. The numerically increased, although non-significant, citric acid values would tend to negate the above argument and advocate the alternative. It is, therefore, concluded that if acetyl-salicylic acid functioned as an anti-stress moiety, it was either independent of the commonly used criterion of adrenal weight or that many more observations of the type employed in this study are needed to substantiate a significant difference in the criteria employed.

The investigation into gross effects of ASA on protein and carbohydrate metabolism was initiated utilizing measurements on blood plasma protein, creatinine, uric acid and liver glycogen. The results of these measurements are given in Table 10 with four replications for the first three criteria and three values for each

Table 10. Blood Plasma Protein, Creatinine, Uric Acid and Liver Glycogen of Hens Fed Levels of Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Plasma protein mgs./100 ml.[a]	Plasma creatinine mgs./100 ml.[a]	Plasma uric acid mgs./100 ml.[a]	Liver glycogen % in liver[b]
None	7.5	2.71	6.67	1.30
0.05	6.5	1.48	6.07	1.55
0.10	6.9	1.68	5.32	0.76
0.20	6.8	3.32	5.00	0.98
0.40	6.0	0.75	4.85	0.33
0.80	6.4	2.91	4.71	0.41
	1.02[c]	0.84	1.19	0.41

[a] Each mean is the average of four replications per determination.

[b] Each mean is the average of three replications per determination.

[c] Pooled estimates of the standard error of the mean.

liver glycogen mean. Variation encountered in these measurements was exceedingly high within replicates with C.V.'s of over forty per cent for each analysis of variance. For this reason the data will be discussed in terms of trends rather than absolute statements, except in the case of liver glycogen. In the latter instance, it was found that considerable reduction in error could be attained by blocking the results on the basis of the day of each complete determination. When this was done, partition of treatment degrees of freedom into orthogonal polynomials indicated that the first four treatments employed produced glycogen values significantly ($P < 0.05$) different from hens fed the highest two levels of drug. Partitioning, according to linear and residual components, indicated that the residual was significantly different from error showing that some type of non-linear curve might fit the data. This point needs more emphasis to establish any definitive type of dose response relationship. Cursory inspection of the data reveals an initial increase and then progressive decreases in percentage liver glycogen content. This initial increase cannot be compared with depletions mentioned in the introduction since this level of drug (0.05%) was lower than employed by other workers. However, the depressions observed are in agreement with the previously discussed work, using other animals, when higher drug levels were employed.

Plasma protein and uric acid levels were both reduced at all levels of drug employed while this was generally, although not specifically, true for plasma creatinine. Even though these results did not approach significance, it would be extremely fortuitous that all parameters would show the same patterns due to chance alone. This notion is further supported by the fact that different samples and hens were used in all determinations. For these reasons, the results are deemed at least promising in supporting the thesis of some reduction in protein metabolism or absorption. Assuming a positive interpretation of the data in vivo, retention per se would not be affected since both plasma protein and its chief excretory product (uric acid) are decreased by feeding the drug. If increased metabolic degradation were involved, the decrease in serum protein observed would be accompanied by increased uric acid values. This would lend substance to the notion of an impairment of protein absorption. A retention study would resolve this argument.

In order to more precisely evaluate these observed effects on carbohydrate and protein metabolism, additional studies were initiated to obtain information at the cellular level. Accordingly, certain enzyme systems were evaluated utilizing liver homogenates obtained from the hens fed increasing amounts of ASA. The results are presented in Table 11 for those systems evaluated in the Warburg apparatus; spectrophotometrically determined systems are given in Table 12.

Table 11. Effect of Dietary Acetyl-Salicylic Acid on Oxygen Uptake by Liver Homogenates for Two Enzyme Systems

Level of dietary acetyl-salicylic acid (%)	Kreb's cycle micro l. O_2 uptake/50 min./50 mg.	Xanthine dehydrogenase micro l. uptake/20 min./100 mg.
None	84.3[a]	61.5[b]
0.05	117.1	61.9
0.10	119.5	58.1
0.20	76.3	58.6
0.40	145.3	62.7
0.80	98.8	64.5
	12.4[c]	9.1

[a] Each mean is the average of five replicates per determination.

[b] Each mean is the average of four replicates per determination.

[c] Pooled estimates of the standard error of the mean.

Table 12. Effect of Dietary Acetyl-Salicylic Acid on Several Spectrophotometrically Measured Liver Enzyme Systems

Level of dietary acetyl-salicylic acid (%)	Cytochrome[a] oxidase	Succinic dehydrogenase	Isocitric dehydrogenase	Glutamic-oxalacetic transaminase
None	74.3[b]	11.5[b]	171.8[c]	56.4[c]
0.05	63.2	7.2	181.6	55.1
0.10	87.1	10.2	177.7	50.7
0.20	67.7	8.7	197.3	57.9
0.40	84.3	10.6	210.3	56.0
0.80	71.0	8.4	203.5	48.5
	10.3[d]	1.7	29.5	5.6

[a] Units = change in optical density per minute per gram.

[b] Each mean is the average of five replicate determinations.

[c] Each mean is the average of four replicate determinations.

[d] Pooled estimates of the standard errors of the mean.

Three enzyme systems associated with carbohydrate metabolism were evaluated. Values obtained for Kreb's cycle activity were quite variable and did not exhibit a particular pattern of oxygen uptake. Since these figures could be considerably distorted by many metabolic factors, two more specific systems were investigated. Again considerable biological variation was encountered. However, it appeared that some increase in isocitric dehydrogenase (ICD) activity was allowed by the ASA levels employed, while all values for succinic dehydrogenase (SD) showed a slight reduction when compared with the value obtained for the control tissue. It appears that the observed differences in the results for SD could be erroneous, since the linear component of treatment sum of squares did not approach significance. However, the increased levels of ICD activity, even with the high mean standard error, appear to be something more than anomalous and may be evidence for increased carbohydrate utilization. These increased activities may partially explain the large decrease in glycogen storage.

Both, glutamic-oxalacetic transaminase (GOT) and xanthine dehydrogenase (XD), were determined to aid in the explanation of the results obtained with the blood samples (Table 10). No evidence was found (Tables 11 and 12) for any appreciable change in either system. Since XD was particularly unaffected by treatment, additional presumptive evidence that decreased uric acid found in blood is related to metabolism of the decreased protein found in this fluid

is available. Values obtained from cytochrome oxidase activities indicated that the treatments did not substantially affect this enzyme. Since this enzyme is near the terminal portion of electron transport, any alteration in metabolism must either be non-existent or this particular molecule was present in such abundance that increased amounts were not necessary to allow increased electron transport. The latter portion of the argument might explain the slight (non-significant) increase ICD observed without a concomitant increase in cytochrome oxidase activity.

Much of the data collected at this point in these studies was relatively inconclusive or contradictory in determining any mode of action for ASA, in either explaining the observed reproductive response to low level feedings of drug or in illuminating a mechanism for the toxic effects observed. It was, therefore, decided that information on the relative blood plasma levels and liver concentrations of ASA might prove of value. The latter organ was selected because of its primary metabolic role and the former to ascertain that increasing dietary amounts of drug were, in fact, being absorbed. Since the gall bladder is attached to the liver, this reservoir of material was also removed and ASA determined on its contents. Additional work of this type is presented in the following chapter. Results are presented in Table 13.

Increasing dietary levels of acetyl-salicylic acid produced concomitant increases in salicylic acid concentrations in the three

Table 13. Plasma, Liver and Bile Concentrations of Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Blood plasma mcg./ml.	Liver mcg./gm.	Bile mcg./ml.
None	---[a]	---	---
0.05	11.4	4.6	1.4[b]
0.10	18.2	14.7	1.0
0.20	49.9	11.7	14.3
0.40	89.4	21.2	53.4
0.80	161.8	36.9	112.3

[a] Values obtained from hens fed the control diet have been subtracted as blank or interfering substances.

[b] Only one determination is involved since a pooled sample obtained from four gall bladders was utilized. Blood plasma means are based on four replicates while liver values are means of three values.

parameters studied. Linear partitions of treatment sums of squares for both plasma and liver salicylic acid were significant. Since a single pooled sample of bile (Table 13) was employed, no ordinary analysis of variance could be attempted. However, regression procedures indicated that the slope was greater than zero ($P < 0.01$). These data indicate that increasing ASA levels produced appropriately increased blood concentrations, thus assuring that proportionally increased amounts of drug could reach the site or sites necessary to elicit the observed reproductive results. The amounts of drug found in the liver were higher than anticipated, but may be partially inflated by blood levels since incomplete bleeding could trap this fluid in an organ with such profuse vascularity as the liver. The amounts found in this organ would be expected to be enough, particularly at the highest level, to inhibit any enzyme systems for which it might be antagonistic. No evidence was found to indicate any profound enzymatic inhibition and only a slight increase in ICD was noted. The observation that bile contained appreciable salicylic acid, particularly at the higher dietary levels, was surprising. No previous work has shown the presence of salicylic acid in this fluid. Particular importance might be attributed to this finding if stimulation or inhibition of bladder evacuation should also occur. Either result would have an effect on fat absorption.

In a last effort to explain the reproductive phenomena observed, it was decided to investigate any possible anti-microbial

properties of acetyl-salicylic acid. Certain early (1800's) workers have attributed antiseptic properties to this compound. Also, the three initial production studies showed that oxytetracycline elicited an analogous stimulation of production to that found for ASA. In the first two studies, feeding a combination of both drugs failed to produce further increases in production. A reduction was encountered in the third trial. These facts would justify pursual of antibacterial properties attributable to ASA. Procedural details are located in the experimental portion of this chapter. Results are tabulated for two collection periods in Table 14.

No particular trends showing any in vivo antibiotic or antiseptic properties for this drug were found. In order to further enhance this notion, sensitivity tests were carried out on isolates from the plates employed. Concentrations of ASA from 10 to 1000 ppm failed to inhibit any isolate while only 0.4 mcg. oxytetracycline per paper disc gave large zones of inhibition. Assuming no adaptations to the drug by the various organisms, then some evidence indicating a non-antimicrobial involvement for ASA has been presented.

Critical examination of the tables in this chapter revealed that some rather large numerical differences were not statistically significant. Since biochemical data, particularly enzymatic activities, are not generally subjected to statistical procedures, calculations were made to illustrate the number of replications necessary to show a significant difference for the largest mean differences observed. The results of these calculations are shown in Appendix A.

Table 14. Microbial Counts on Cecal Contents of Hens Fed Levels of Dietary Acetyl-Salicylic Acid

Level of dietary acetyl-salicylic acid (%)	Total count nos./gm. x 10^6		Coliforms nos./gm. x 10^5		Yeast nos./gm. x 10^2	Molds nos./gm. x 10^a	Lactics nos./gm. x 10^6
	1	2	1	2	1	1	1
None	28	58	199	122	16	5	333
0.05	0	663	5	687	55	22	1253
0.10	6	259	65	239	16	13	227
0.20	17	10	27	5	144	6	20
0.40	4	394	356	443	14	19	493
0.80	10	77	56	29	129	7	113

[a] Numerals one or two indicate collection periods 1 and 2, respectively.

Summary. Four experiments were conducted showing that 0.05% dietary acetyl-salicylic acid (ASA) significantly increased egg production and improved efficiency of food utilization in laying hens. These production increases were independent of temperature, and hence of the antipyretic action of ASA. Variable effects were observed on shell percentage while the drug was without effect on other parameters studied. In one experiment dietary levels of up to 0.20% produced analogous results. The same study showed that a high level of ASA (0.80%) significantly depressed egg production, feed utilization and intake, shell quality and body weight gain. Inclusion of oxytetracycline in the ration (25 gms./ton) produced results similar to those found for 0.05% ASA. In two studies, a combination of both drugs failed to increase egg production or further enhance the other factors studied when compared to results for either drug fed alone. In a third trial, the combination of drugs allowed results no different from those obtained from the control hens.

In an effort to explain the aforementioned changes, certain biochemical measurements were made. The following points were discussed:

1. Visceral fat and adrenal weights were depressed when 0.80% ASA was fed. However, these depressions were not independent of current bird weight.

2. The fatty acid composition of abdominal and liver lipids was unchanged by dietary levels of ASA, indicating no metabolic error in fat metabolism, unless it were one of rate limiting deposition.

3. Plasma ascorbic acid was numerically decreased while citric acid was numerically increased by the dietary increments of ASA employed. Therefore, the observed decreased in adrenal size could not be resolved via these measurements.

4. Plasma protein, creatinine and uric acid were numerically depressed by all levels of ASA indicating some reduction in protein metabolism or absorption. Liver glycogen was significantly lowered at the two highest levels of dietary drug.

5. Six liver enzyme systems were examined. Isocitric dehydrogenase was positively affected by dietary ASA perhaps explaining some of the observed decrease in glycogen storage while no differences were observed in the other systems studied.

6. Blood plasma levels of ASA were increased with increasing dietary drug levels. Liver was similarly affected. Unexpectedly, large amounts of drug were found in bile, which may have had some effect on fat absorption.

7. Microbial counts and sensitivity tests on isolates from cecal contents revealed no antimicrobial or antiseptic properties attributable to the levels of drug employed.

CHAPTER IV

SOME BIOCHEMICAL AND PRODUCTIVE EFFECTS OF FEEDING ACETYL-SALICYLIC ACID AND CHEMICAL ANALOGS TO CHICKS

Experimental. New Hampshire x Delaware chicks were used in four experiments to determine the effects of feeding acetyl-salicylic acid (ASA) on chick growth and feed utilization. Two additional experiments, using similar chicks, were conducted to evaluate the same effects with chemical analogs of ASA on identical parameters and to obtain experimental units for biochemical evaluations. Chicks in the first three studies were grown to four weeks of age in electrically-heated battery brooders on raised wire floors. In the fourth study, chicks were grown on straw litter to eight weeks of age. In the final two studies, the same type of chicks were fed the dietary treatments for two weeks beginning at 28-days of age. These birds were housed in growing batteries with raised wire floors. No supplemental heat source was supplied. The compositions of the basal diets are shown in Table 15. Both feed and water were supplied ad libitum.

The samples obtained for biochemical measurements were taken from birds at the termination of the last two experiments. Blood plasma samples were obtained by cardiac puncture, while organ samples were excised from decapitated birds. The complete brain was removed

Table 15. Composition of Experimental Diets for Chicks

Ingredients	Experiments 1, 2, 3, 4[a] (%)	Experiments 5 and 6 (%)
Ground yellow corn	50.00	61.00
Dehydrated alfalfa meal (17% protein)	2.00	2.00
Soybean meal (44% protein)	31.28	21.50
Fish meal (65% protein)	5.00	4.00
Meat and bone scraps (50% protein)	-	2.00
Animal fat	5.00	3.00
Dried whey	1.00	1.00
Distillers dried solubles	1.00	-
Dicalcium phosphate	1.00	1.00
Calcium carbonate	0.75	1.50
Sodium chloride	0.35	0.40
Manganese sulfate pentahydrate (70%)	0.02	0.02
Methionine hydroxy analog	0.10	0.05
Vitamin premix[b]	2.50	2.50
	100.00	99.97

[a] This diet was employed the first four weeks of Experiment 4. During the final four weeks the birds were fed the second diet.

[b] Supplied the following per pound of diet: 4500 I.U. vitamin A, 700 I.C.U. vitamin D_3, 5.0 mg. calcium pantothenate, 400 mg. choline chloride, 60 mcg. vitamin B_{12}, 2.5 I.U. vitamin E, 1.0 mg. vitamin K, 56.75 mg. ethoxyquin (as a preservative) and 8.4 gms. soybean meal as a carrier.

by cutting through the upper cranial material with an electric-powered all-purpose tool equipped with a rotary blade. The procedures employed in the various analysis were the same as those discussed in the previous chapter with the exception of blood glucose and packed cell volume (hematocrit). Blood glucose was determined on protein-free plasma samples (see Chapter III) using a modification of the method suggested by Morris (1948), while hematocrits were obtained by using capillary tubes for the measurement of the packed cell volumes after centrifugation. Statistical procedures were the same as those employed for the hen studies.

Results and Discussion. Acetyl-salicylic acid (ASA) fed at levels of 0.005-0.08% of the diet failed to produce a significant difference in growth rate or feed conversion in the three studies conducted in starting batteries (Table 16). Levels of up to 0.075% ASA were fed to broiler chicks for eight weeks, and no significant improvements in growth rate or feed conversion were obtained with these birds housed on litter (Table 17). In an additional study (Experiment 5), similar results were obtained with increments of ASA from 0.05 to 0.30% (Table 18). A single level of methylene-disalicylic acid (MDS), equivalent (on a percentage salicylic acid basis) to the highest level of ASA employed (0.30%), failed to have a statistically significant effect on either growth (gain) or feed utilization. The final experiment involved the feeding of ASA, MDS, para-amino-salicylic acid (PASA) and

Table 16. Effect of Acetyl-Salicylic Acid on Body Weight and Feed Conversion of Chicks at 4 Weeks

Supplement to basal diet	Experiment 1[a]		Experiment 2[a]		Experiment 3[b]	
	Body weight (gms.)	Feed/ unit gain	Body weight (gms.)	Feed/ unit gain	Body weight (gms.)	Feed/ unit gain
None	394	1.91	426	1.68	399	1.58
0.005% acetyl-salicylic acid					401	1.57
0.010% acetyl-salicylic acid					398	1.57
0.015% acetyl-salicylic acid					395	1.65
0.020% acetyl-salicylic acid			435	1.62	380	1.68
0.025% acetyl-salicylic acid						
0.040% acetyl-salicylic acid			453	1.52		
0.050% acetyl-salicylic acid	368	1.93				
0.075% acetyl-salicylic acid						
0.080% acetyl-salicylic acid			426	1.59		

[a] Each dietary treatment was fed to four replications of 8 chicks each.

[b] Each dietary treatment was fed to four replications of 9 chicks each.

Table 17. Effect of Acetyl-Salicylic Acid on Body Weight and Feed Conversion of Chicks at 4 and 8 Weeks

Supplement to basal diet[a]	4 Weeks		8 Weeks	
	Body weight (gms.)	Feed/ unit gain	Body weight (gms.)	Feed/ unit gain
None	407	1.74	985	2.27
0.025% acetyl-salicylic acid	397	1.75	994	2.15
0.050% acetyl-salicylic acid	392	1.76	971	2.19
0.075% acetyl-salicylic acid	297	1.72	1003	2.10

[a] Each dietary treatment was fed to 2 pens of New Hampshire x Delaware chicks containing 31 males and 31 females each.

Table 18. Effect of Acetyl-Salicylic Acid and Similar Compounds on Body Weight Gain and Feed Conversion of Chicks from 4 to 6 Weeks of Age

Supplement to basal diet (%)	Experiment 5[1]		Experiment 6[2]	
	14-day body wt. gain (gms.)	Feed/ unit gain	15-day body wt. gain (gms.)	Feed/ unit gain
None	361	2.66	446[b][7]	2.98
0.05 ASA[3]	367	2.47	--	--
0.10 ASA	350	2.47	--	--
0.15 ASA	370	2.54	--	--
0.20 ASA	377	2.54	--	--
0.25 ASA	353	2.56	--	--
0.30 ASA	358	2.34	--	--
0.40 ASA	--	--	432[b]	2.96
0.24 MDS[4]	37[illegible]	2.51	--	--
0.32 MDS	--	--	464[ab]	2.60
0.34 PASA[5]	--	--	444[b]	2.43
0.335 APAP[6]	--	--	489[a]	2.49

[1] Each dietary treatment was fed to two replications of 8 chicks each.

[2] Each dietary treatment was fed to 8 chicks. Birds were weighed and statistically analyzed individually.

[3] ASA = acetyl-salicylic acid.

[4] MDS = methylene disalicylic acid.

[5] PASA = para-amino-salicylic acid.

[6] APAP = acetyl-para-amino phenol.

[7] Averages having different superscripts are significantly different at the 0.05 level of probability.

acetyl-para-amino phenol (APAP). These chemical analogs, excepting APAP, were fed at a rate such that their dietary salicylic acid contribution was equivalent to that found in the 0.40% ASA diet. The diet containing APAP was fed at the phenol equivalent of the 0.40% ASA feed. The results (Table 18) indicate a significant growth promoting property for APAP, when compared to control fed birds, while the other drugs had no effect. In both Experiments 5 and 6, the results obtained indicated a numerically superior gain for birds fed each of the two levels of MDS when these gains were compared to those obtained with chicks fed the control diet.

Even though growth responses were not obtained with ASA or the other drugs employed, excepting APAP, additional biochemical evaluations were made on birds from Experiments 5 and 6 in the hope that such data might aid in the interpretation of the results discussed in Chapter III. Accordingly, plasma salicylic acid and liver glycogen were measured. The results for both experiments appear in Table 19.

Plasma salicylic acid values were increased with each dietary increment of ASA employed in Experiment 5. However, these increases were not in a particularly good dose response relationship, as was the case in the laying hen studies (Chapter III). This lack of a completely linear fit is probably due to the narrowness of the drug levels employed (increments of 0.05%), and also the feed intake exhibited by these birds was approximately two-thirds that found for

Table 19. Plasma Salicylic Acid and Liver Glycogen Contents of Chicks Fed Acetyl-Salicylic Acid and Other Similar Compounds

Supplement to basal diet (%)	Experiment 5		Experiment 6	
	Plasma salicylic acid mcg./ml.	Liver glycogen (%)	Plasma salicylic acid mcg./ml.	Liver glycogen (%)
None	--[2]	0.6	0.5[3]	0.5[a4]
0.05 ASA[1]	8.7	1.3	--	--
0.10 ASA	19.9	0.9	--	--
0.15 ASA	35.5	0.9	--	--
0.20 ASA	36.4	0.6	--	--
0.25 ASA	47.1	1.1	--	--
0.30 ASA	48.9	1.4	--	--
0.40 ASA	--	--	43.7	0.6[ab]
0.24 MDS	0.3	0.8	--	--
0.32 MDS	--	--	2.9	1.4[b]
0.34 PASA	--	--	19.6	0.9[a]
0.335 APAP	--	--	0.6	1.0[ab]
	5.01[5]	0.23	3.12	

[1] See Table 18 for key to abbreviations.

[2] A mean of zero for the four replicates was actually determined.

[3] Two replications per mean.

[4] Averages having different superscripts are significantly different at the 0.05 level of probability.

[5] Pooled estimates of the standard error of the mean.

the laying hens. Of particular interest was the low level of plasma salicylic acid exhibited by the birds fed 0.24% MDS. This depressed value was not anticipated, even though the chemical procedure employed was specific for only salicylic acid, since it was assumed that this drug would be hydrolyzed after or during absorption. Obviously, this was not true. The results of plasma salicylic acid analysis in Experiment 6 were similar with reference to these two drugs. In addition, APAP was found in insignificant amounts. This was not unexpected since the method employed would not be expected to detect this drug. The level of salicylic acid found with PASA was about half of that determined in plasma of chicks fed an equivalent amount of ASA. Since the method was known to be approximately 80% efficient in detecting this compound, approximately 30% of PASA was either unabsorbed, quickly degraded or excreted.

Liver glycogen was numerically increased in both experiments at each increment of ASA fed. With the exception of the 0.20% level, there was no evidence for a marked reduction at higher dietary drug levels as was found with hens. The other drugs employed markedly increased storage of this compound in Experiment 6. Glycogen in livers from chicks fed MDS was significantly greater than the percentage found in the organs from birds fed the control diet. Neither of the two factors studied have any obvious relation to the growth or feed utilization data obtained. Also, they do not completely agree with the laboratory results obtained from determinations on blood and livers of hens fed ASA levels. Since uric acid was

numerically depressed in plasma of hens fed ASA levels, it was decided to evaluate blood obtained from Experiment 5 for this constituent. Blood glucose was also estimated on the same filtrates to attempt an explanation for the numerically increased glycogen stores. The results are in Table 20.

Again, as in the hen study, uric acid values were numerically depressed when compared to the result obtained from chicks fed the control diet. A similar result was obtained with the single level of MDS employed. While these results in themselves are not conclusive, the fact that a similar outcome existed in the hen study would suggest that depressed uric acid is a real metabolic effect of dietary ASA. It would be presumptive, with only one dietary level employed, to imply the same effect for MDS.

Plasma glucose levels were significantly greater in all treated groups, when a single treatment degree of freedom was used to compare all means to the control. These increased values were interpreted concomitantly with the observed increased liver glycogen values (Table 19). The most plausible explanation would involve a total increase in gluconeogenesis providing increased storage. This increase could have also been accompanied by decreased protein catabolism since uric acid values were also depressed.

One major problem encountered in the hen studies was some confusion in interpreting reduced organ weights produced by high dietary levels of ASA when this drug also depressed body weights.

Table 20. Plasma Uric Acid and Glucose of Chicks Fed Levels of Acetyl-Salicylic Acid

Supplement to basal diet (%)	Plasma glucose mgs./100 ml.	Plasma uric acid mgs./100 ml.
None	138.1[b]	4.26
0.05 ASA[a]	206.2	3.83
0.10 ASA	283.7	3.49
0.15 ASA	169.8	3.65
0.20 ASA	165.4	3.90
0.25 ASA	326.1	3.25
0.30 ASA	161.2	3.45
0.24 MDS	184.9	3.88
	38.8[c]	0.38

[a] See Table 18 for key to abbreviations.

[b] Both determinations involved four replicates per mean.

[c] Pooled estimates of the standard error of the mean.

Since only one significant difference in body weight was detected in Experiment 6, it was deemed productive to excise organs from these birds to evaluate any drug effects on organ weight which would be independent of body weight. Weights obtained for liver, spleen and kidney revealed no numerically meaningful differences. It was not possible to obtain enough visceral fat for weight evaluation from any treatment group. Hematocrit values obtained from these same birds revealed no significant or numerical effects attributable to treatments.

Some effort was made in the previously discussed hen experiments to evaluate tissue levels of salicylic acid. This approach was again employed for salicylic acid determinations on brain and kidney tissue obtained from birds in Experiment 6. In addition, bile was again examined in order to determine if this fluid might serve as a major excretory route in the chick as was suggested by the hen data. Results are presented in Table 21.

The amount of salicylic acid found in gall bladders was again substantial. No statistical analysis of these data was possible since four individual bladders per treatment group were pooled to obtain enough fluid for evaluation. The volume of bile in these bladders is also given in Table 21. No differences were evident for this criterion. These data suggest that <u>in vivo</u> hydrolysis of MDS had apparently taken place since salicylic acid was quite high in bile from birds fed this drug. It was previously

Table 21. Salicylic Acid Contents of Kidney, Brain and Bile of Chicks Fed Acetyl-Salicylic Acid and Some Chemical Analogs

Supplement to basal diet (%)	Bile mcg./ml.	Brain mcg./gm.	Kidney mcg./gm.	Bile volume ml./bladder
None	--[b]	--[b]	--[b]	0.41[c]
0.40 ASA[a]	8.3	3.47	9.40	0.40
0.32 MDS	14.6	--	2.50	0.42
0.34 PASA	3.8	1.24	3.40	0.29
0.335 APAP	1.5	--	--	0.33
				0.11[d]

[a] See Table 18 for key to abbreviations.

[b] Values obtained with no dietary drug were subtracted as blank from the other values.

[c] There were four replicate measurements per mean.

[d] Pooled estimate of the standard error of the mean.

indicated that the method employed was not specific for MDS. It should be noted that the blood of these chicks contained only 2.9 mcg. salicylic acid/ml. (Table 19). Brain tissue from MDS treated chicks contained no detectable salicylic acid, while kidney tissue contained 2.5 mcg./gm. Undoubtedly, part of this latter amount was due to blood drug level in addition to some hydrolysis in the kidney. Apparently no hydrolysis of MDS occurred in brain, since salicylic acid was not found in this tissue. Bile and tissue salicylate contents of birds fed ASA were lower than anticipated. However, the numerical similarity of bile and kidney tissue contents might support a view of equal elimination via kidney and bile. Since it is known that conjugation products of salicylic acid occur prior to elimination from kidney and since dissimilar units of bile and kidney concentrations were employed, a more likely hypothesis would be for some bilary excretion of salicylic acid with a major route through the kidneys. Since the ratio of excretory routes for MDS (at least as salicylic acid) is altered towards bile, this may be a more significant route of elimination than that discussed for ASA. The same ideas advanced for ASA seem to explain the observed distribution for PASA. Values observed were lower than expected, probably for the reasons previously advanced in the discussion of results found in Table 19. The small amount of APAP found in bile would indicate some excretion as salicylic acid via this route. However, no drug was found (as salicylic acid) in brain or kidney.

Summary. Six experiments were conducted showing that from 0.005 to 0.40% dietary acetyl-salicylic acid (ASA) did not significantly affect growth or feed conversion of chicks housed in several environments and at three different ages. In two experiments, numerically increased growth was observed for birds fed methylene-di-salicylate (MDS). The results of a final experiment, utilizing chemical analogs of ASA, showed that a significant growth promoting effect was obtained with 0.35% dietary acetyl-para-amino phenol (APAP). One other drug, para-amino-salicylic acid (PASA), was not effective as a growth promoting substance.

Even though notable growth responses were not observed, it was thought desirable to duplicate and perhaps extend some of the biochemical determinations evaluated during the hen studies as an additional aid in evaluating those results. The following points were discussed in relation to the hen studies:

1. Similar increases in plasma salicylic acid were observed when increasing dietary levels of ASA were fed. These increases were not absolutely linear. A very small recovery for MDS (as salicylic acid) was noted while other drugs were intermediate in increasing blood plasma salicylic acid content. Liver glycogen was numerically increased by all dietary treatments as contrasted to significantly depressed values obtained from hens fed high drug levels (0.40 and 0.80% ASA).

2. All dietary treatments decreased plasma uric acid (as noted for hens) and significantly increased glucose again implicating decreased protein metabolism as a real metabolic effect for ASA. Increased plasma glucose may explain the higher liver glycogen values observed for the treated groups.

3. Organ weight measurements, uninfluenced by body weight, showed no differences attributable to treatments.

4. Bile was again implicated as a route of elimination for salicylic acid. Apparently hydrolysis of MDS occurred in liver and kidney since this drug was found, as salicylic acid, in both bile and kidney. No differences in bile volume were noted. ASA was also found as salicylic acid in both bile and kidney with more appearing in the latter organ than was found when chicks were fed MDS.

Appendix A. Replications Necessary to Detect Several of the Largest Mean Differences Tabled in the Text

Table no.	Section	C.V.[1]	Observed largest mean difference	Grand mean	Error degrees of freedom	Number of replications necessary[4]
7	Palmitic acid	9.00	1.53	20.03	12	23
7	Oleic acid	6.16	3.37	51.10	12	15
8	Palmitic acid	14.34	6.89	27.34	6	6
8	Oleic acid	14.62	6.98	47.95	6	17
9	Citric acid	27.46	15.63	41.46	12	9
10	Uric acid	43.44	1.96	5.44	18	23
10	Glycogen	80.46	1.22	0.89	12	20
11	Xanthine dehydrogenase	29.57	6.40	61.20	18	126
12	GOT[2]	20.57	9.40	54.10	18	23
12	ID[3]	24.85	38.50	190.34	15	25

[1] Coefficient of variation.

[2] Glutamic-oxalacetic transaminase.

[3] Isocitric dehydrogenase.

Where: T_0 = Tabled T, type I error

T_1 = Tabled T, type II error

X = Percentage of mean difference

[4] Calculated from: $n = 2(T_0 + T_1)^2 \left(\frac{C.V.}{X}\right)^2$, applied three times.

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