THE EFFECTS OF POLYBROMINATED BIPHENYLS ON THE HEMATOLOGY AND PLASMA ERYTHROPOIETIN LEVELS OF THE SINGLE COMB WHITE LEGHORN COCKEREL

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LINDA RAE VAN THIEL

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By

Linda Rae Van Thiel

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ABSTRACT

THE EFFECTS OF POLYBROMINATED BIPHENYLS ON THE HEMATOLOGY AND PLASMA ERYTHROPOIETIN LEVELS OF THE SINGLE COMB WHITE LEGHORN COCKEREL

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Polybrominated biphenyls (PBB), a thermoplastic fire retardant commercially available as Fire Master FF-1, was accidentally incorporated into livestock feed in the State of Michigan. PBB and related compounds, have been found to alter the hematocrits and hemoglobin concentrations of contaminated animals. The purpose of this study was to determine the extent of the hematological disturbance, and to determine whether or not the disturbance was accompanied by a change in plasma erythropoietin (ESF) levels in the single comb white leghorn (SCWL) cockerel.

In the first experiment, 27 SCWL cockerels were divided into three equal groups. One group was fed rations containing 150 ppm PBB. The two control groups were fed rations containing 0 ppm PBB; one group was fed ad libitum, the other was pair-fed to the 150 ppm PBB group to eliminate any possible effects due to any decrease in feed consumption. The second experiment was similarly designed using 50 week-old cockerels. These birds were divided into two equal groups and were fed either 0 or 150 ppm PBB. Due to limited facilities the ad libitum control group was

eliminated in this experiment allowing for maximum numbers of birds in the pair-fed control and the treated groups.

The ESF levels were determined by bioassay using Japanese quail. In the first and second experiments, respectively, 50 and 60 adult males were exposed to hypoxic conditions daily for three weeks. This treatment increased their sensitivity to any plasma ESF present in the serum prepared from the cockerels fed 0 or 150 ppm PBB.

After 4½ and 8 weeks of feeding in the first experiment, and after 8 weeks of feeding in the second experiment, statistically significant differences were observed between the control and PBB treated group mean hematocrits and hemoglobin concentrations. Both hematological values were decreased in the treated group versus the control groups. There was also a significant difference between the mean ESF level of the control and treated groups. The plasma ESF level of the treated group was lower than that of the control group. The results of these studies indicated that the decrease in hematological values due to chronic ingestion of PBB was the result of depressed levels of plasma ESF.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Polyhalogenated biphenyls have been commercially available since the early 1930's. Polychlorinated biphenyls (PCBs) have been recognized as an environmental contaminant since 1966. Recently, measures have been introduced to reduce the amounts of PCBs released into the environment.

Polybrominated biphenyls (PBBs) have been realized as an environmental contaminant in Michigan only within the last few years. PBBs were developed and produced by the Michigan Chemical Company as a fire retardant for thermoplastics under the name of Fire Master FF-1. In May 1973, an unknown number of 50 pound bags, estimated at a total of several hundred pounds, were mistakenly received by the Michigan Farm Bureau. The Farm Bureau routinely mixes a nutritional supplement, magnesium oxide sold as Nutrimaster, into its feeds. Prior to 1973, the consistency of Fire Master and Nutrimaster were quite different, but due to additional processing of the Fire Master, the compounds looked like identical fine, white powders. In addition, due to a paper shortage in 1973, both compounds were packaged in identical bags, with only the name of the compound stenciled across each bag. The mistake in shipment was not realized and the Fire Master FF-1 was incorporated into livestock feed. Subsequently, thousands of Michigan beef and dairy cattle, sheep, swine, poultry, and indirectly, humans, were contaminated with unknown

amounts of PBB. PBBs were not implicated as the source of the contamination until the following year.

Gross symptoms of polyhalogenated biphenyl contamination are skin lesions, increased skin pigmentation, edema, loss of appetite, alterations in reproduction, and liver damage. Over a moderate length of time, PCBs cause a noticeable anemia in fowl. It has since been found that PBB intoxication also results in anemia. The purpose of this study was to determine whether or not PBB altered certain hematological values, including hematocrit, hemoglobin concentration and erythropoietin levels in single comb white leghorn cockerels.

LITERATURE REVIEW

Erythropoietin

For many years it has been known that certain conditions alter the number of circulating erythrocytes. Hypoxia or hyperoxia were thought to influence red cell numbers by the direct action of O_2 concentration on the bone marrow. In 1950 Reissman studied parabiotic partially nephrectomized rats and determined that changes in erythrocyte number were due to a humoral factor produced outside the bone marrow. This humoral factor must normally be present, at least intermittently, for red cell number homeostasis, but until recently the normal concentration was too minute to determine by bioassay. Most erythropoietic studies involve animals with elevated hormone levels induced by chemicals, drugs or environmental changes (Jacobson and Doyle, 1962). Along with elevated plasma erythropoietin (ESF) levels researchers found detectable amounts of ESF in urine, lymph, amniotic fluid and possibly breast milk. It was assumed that such fluids normally contain small amounts of ESF as well (Krantz and Jacobson, 1970).

Complete purification of active ESF has not yet been accomplished, although a fraction that is 930,000 times as effective as the original has been attained by extraction on an ion exchange resin at low pH and low NaCl concentration. ESF for purification may be obtained from the urine or blood of patients and animals with ESF levels elevated as a result of severe anemia or chemical treatment. The highly active

preparation was composed of 71% protein and 29% carbohydrate, but approximately two-thirds of the total was assumed to be impurities.

ESF appears to be a protein associated with a glycoprotein. Dukes et al. (1975) have proposed that the glycoprotein protects the hormone from inactivation and provides for target cell specificity. ESF is relatively stable under normal conditions, retaining its biological activity after boiling for 15 minutes at pH 5.5. Mammalian ESF is destroyed by neuraminidase and such proteolytic enzymes as trypsin, pepsin, α-and β-amylase, β-glucosidase, cellulase, ribonuclease, carboxypeptidase and lysozyme. Sialic acid is necessary for its activity. Estimates of molecular weight range from 27,000 to 66,000 with an accepted average value of 46,000 (Nakao et al., 1975).

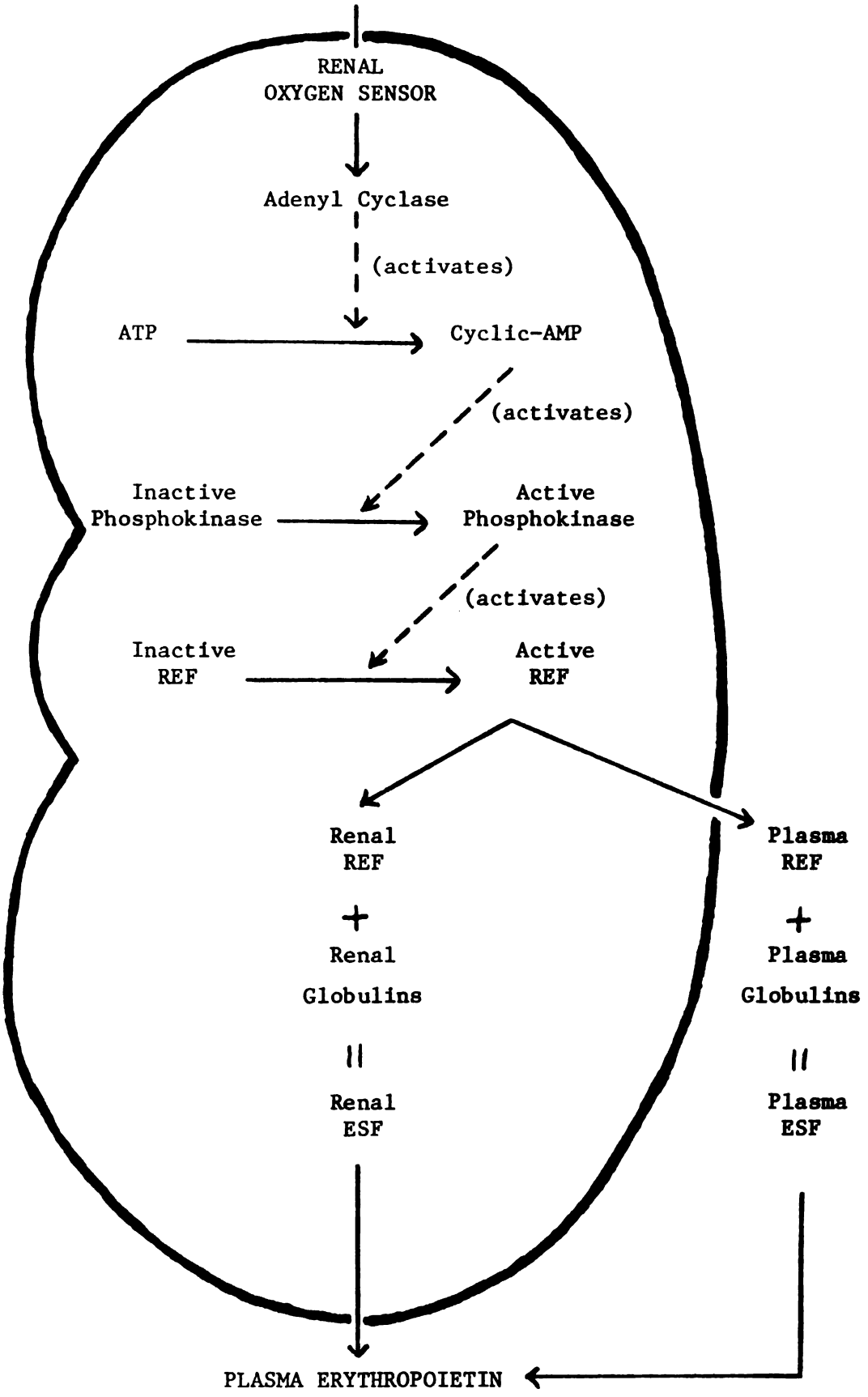
Early studies attributed the majority of ESF production to the kidneys (Jacobson, 1957). Reissman and Nomura (1962) thought the renal medulla was the source of ESF, while others proposed renal ESF was produced in the renal cortex. In 1965, Fisher and coworkers, using fluorescent antibody techniques and electron microscopy, localized the epithelial cells of the glomerulus as the major source of ESF. However, approximately 10% of ESF circulating is thought to be of extra-renal origin, produced by the liver (Reissman and Nomura, 1962), and possibly spleen, pituitary and/or blood vessels (Fisher et al., 1962).

George and coworkers (1975) and Fisher (1975) postulated that cyclic-AMP is the intracellular mediator controlling ESF production in the kidney (Figure 1). They proposed that a renal oxygen sensor detects changes in O_2 concentration due to any one of many stimuli. They also propose that the sensor may be located in glomerular epithelial cells, or it may be in another region of the kidney which in turn stimulates

Figure 1. The proposed intracellular mechanism controlling the production of erythropoietin in a renal glomerular epithelial cell.

ESF = Erythropoietic Stimulating Factor

REF = Renal Erythropoietic Factor


RENAL
OXYGEN SENSOR
Adenyl Cyclase
(activates)
ATP
Cyclic-AMP
(activates)
Inactive
Phosphokinase
Active
Phosphokinase
(activates)
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REF
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REF
Renal
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Renal
Globulins
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PLASMA ERYTHROPOIETIN

Figure 1.

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the glomerulus to produce ESF. The primary stimulus acts on the renal oxygen sensor to activate adenyl cyclase. This membrane-bound compound initiates the conversion of intracellular ATP to cyclic-AMP. Cyclic-AMP activates a protein kinase which in turn activates a renal erythropoietic factor (REF). A REF was isolated in two separate laboratories; both Hansen (1963) and Kuratowska (1968) reported extremely erythropoietic low activity in their factors. Kuratowska proposed that the less active REF and α-globulins, of renal or humoral origin, combine to form active ESF. Fisher et al. (1975) found that the action of the renal sensor appeared to be potentiated by prostaglandins. The prostaglandins PGE_1 and PGE_2 may enhance the renal adenyl cyclase conversion of ATP to cyclic-AMP.

Erythropoietin exhibits its effects on the bone marrow to initiate production of red cells. The exact morphological site upon which the ESF acts has not been identified. ESF seems to stimulate an unidentified stem cell to proliferate and produce erythrocytes. This stem cell, as defined by Gurney (1965), precedes the earliest characterizable erythroblast and differentiates into an erythroblast in the presence of erythropoietin.

The primary regulator of ESF production is the O_2 concentration at the renal sensory cell. A change in O_2 concentration may result from alterations either in atmospheric O_2 pressure, metabolic rate, erythrocyte O_2-carrying capacity, erythrocyte number, or renal blood flow (Figure 2). Blood O_2 concentration is directly affected by hypoxia or hyperoxia; the former stimulates ESF production and the latter depresses it. Administration of cobalt is widely used to elevate ESF levels within a few hours. Cobalt has no effect on overall metabolic

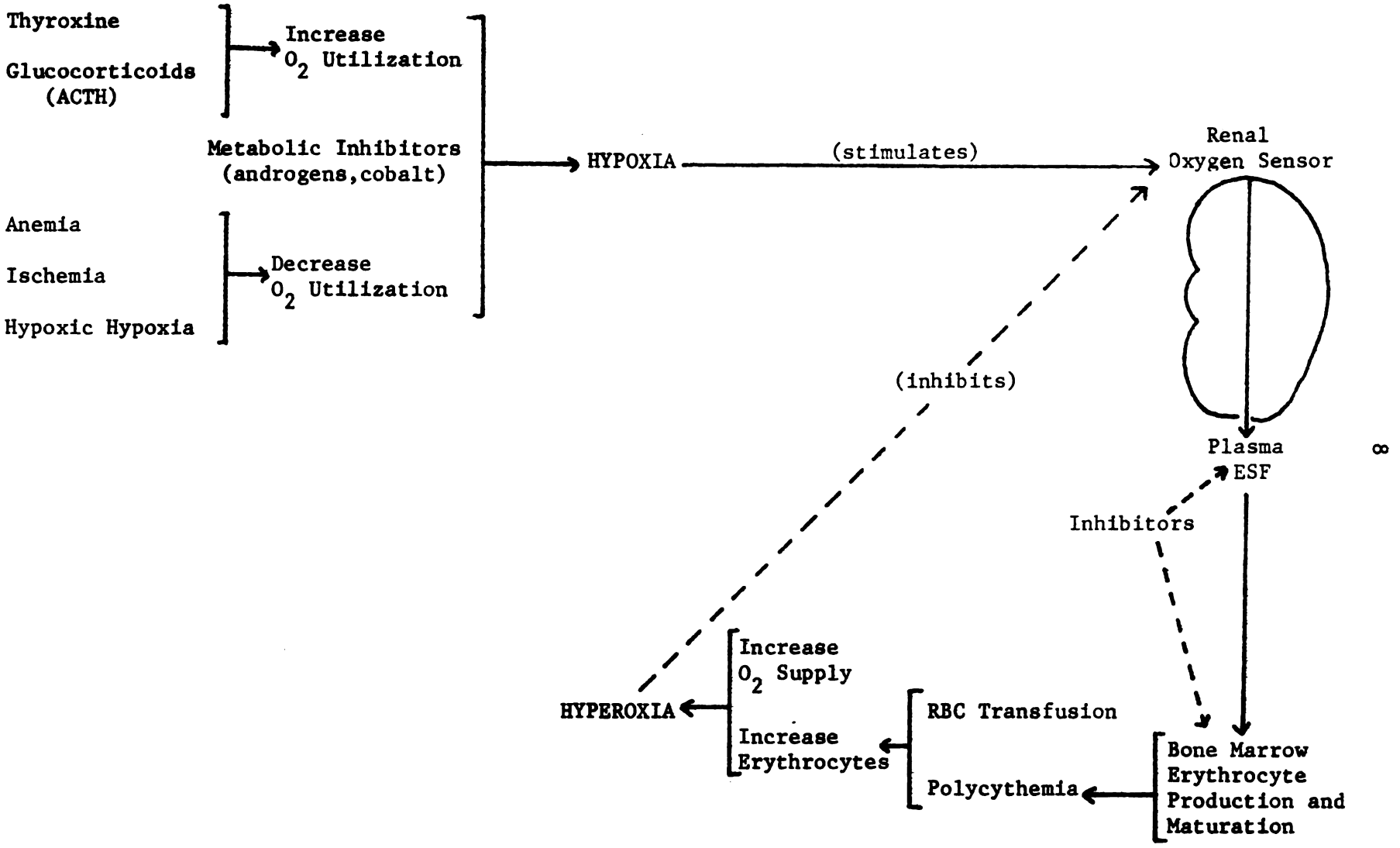


Figure 2. Factors controlling production of erythropoietin (ESF).

rate or O_2 concentration in animals, but directly reduces the oxygenation of renal tissue. The precise mechanism of cobalt action is unknown.

The effect of androgens upon erythropoiesis is a slight increase in red cell numbers over a long period of time. Van Dyke and coworkers (1954) first determined that the gonads were not responsible for ESF production. Many researchers thought testosterone merely potentiated the effects of ESF. It is now known that testosterone directly alters ESF levels, although the mechanism is unknown. McCullagh (1942) postulated that androgens may act as an anabolic stimulus to increase metabolic rate. Gurney (1965) proposed that androgens exert an additional effect directly on the kidneys to increase ESF.

Within an individual species, the greater number of red cells in males than in females is not solely due to the lack of androgen. The estrogens exhibit an anti-androgenic action which additionally decreases erythropoiesis. Mirand and Gordon (1966) found that large doses of estradiol directly inhibited renal ESF production. Dukes and Goldwasser (1961) and Jepson and Lowenstein (1966) found that small doses of estradiol inhibited erythrocyte formation without changing plasma ESF levels. Therefore, small amounts of estrogen may act directly on the bone marrow to depress erythropoiesis while large doses may act through altering ESF synthesis.

The pituitary hormones, adrenal corticotropic hormone (ACTH), growth hormone (GH), and thyroid stimulating hormone (TSH), increase erythropoietic activity. These hormones do not act directly on the erythropoietic system but influence ESF levels by altering metabolic rate and O_2 consumption.

Thyroid hormones, T_3 and T_4, seem to increase erythropoiesis indirectly by increasing O_2 consumption and metabolic rate (Jacobson et al., 1959). Conflicting reports indicate that the degree of change in ESF is not proportional to the change in metabolic rate, although both always change in the same direction. (Donati et al., 1964). In addition, thyroid hormones may have a direct influence either on the kidney or on renal blood flow, but there is no experimental evidence to indicate this action (Krantz and Jacobson, 1970).

Adrenal hormones seem to both stimulate and depress the erythropoietic system, depending on the status of the animal and dosage of the hormone. Patients with adrenalectomies or adrenal cortical dysfunction exhibited a mild anemia (Van Dyke et al., 1954), although actual levels of ESF in such conditions were not determined. Administration of adrenal corticosteroids (AC steroids) increase the metabolic rate of hypophysectomized animals (Evans et al., 1964). Therefore, adrenal hormones may act to influence erythropoiesis by changing the ratio of oxygen supply to oxygen demand.

Stimulation of the central nervous system also affects erythropoiesis. Feldman and coworkers (1966) found that electrical stimulation of the posterior hypothalamus and midbrain of rats increased erythropoietic activity. Mirand et al. (1967) stimulated the supraoptic nucleus, preoptic nucleus, and posterior median eminance of monkeys and noted increased plasma ESF levels. They also proposed that the changes in erythropoietic activity were not a result of changes in pituitary hormones. Takaku et al. (1961) postulated that stimulation of the nervous system, through renal innervation of the splanchnic nerve, changed renal blood flow.

The plasma half-life of ESF has been determined by many researchers and averages 3 to 5 hours in rats. The primary source of ESF elimination is renal excretion, which is roughly proportional to serum levels of ESF. In 1968, Kuratowska isolated a renal ESF inhibiting factor from kidney extracts. It was thermolabile, unaffected by trypsin inhibitor and seemed to be associated with neuraminidase activity. Dukes and coworkers (1972) considered that prostaglandins were an integral cofactor necessary for ESF activity. They isolated a protein urinary ESF inhibiting factor which exhibited a higher affinity for the prostaglandin cofactor than did active ESF. The inhibiting factor was thought to be ESF altered by degradation or faulty synthesis. In 1975, Dukes proposed another mechanism through which ESF inhibition may be mediated by prostaglandins. Certain lipids, possibly other prostaglandins, combine with ESF, replacing normally bound prostaglandins, thus inactivating the complex.

After studying increases in ESF levels in patients exhibiting liver damage due to phenylhydrazine, Jacobsen and coworkers (1956) postulated the metabolism of ESF in the liver. Mirand and Prentice (1959) caused acute liver damage with carbon tetrachloride and found greatly elevated ESF levels. They felt the liver damage lowered the rate of hepatic ESF inactivation. In 1962, Burke and Morse perfused rat plasma having high ESF levels through normal livers and found significantly reduced plasma ESF levels after 2 to 3 hours. Livers pretreated with carbon tetrachloride and perfused with high ESF plasma did not significantly reduce the plasma ESF levels.

Administration of certain chemicals and drugs alters the ability of the liver to produce hepatic microsomal enzymes responsible for

metabolizing various proteins. Smuckler and Benditt (1965) showed that carbon tetrachloride-treated rat liver cells contain hepatic microsomes and ribosomes with a reduced capacity for protein production. Therefore, the enzymes necessary for protein, or ESF, metabolism are not produced with sufficient activity. Conversely, administration of phenobarbital results in induction of many metabolic enzymes (Conney, 1967). Roh and Fisher (1971) perfused plasma ESF through the livers of control dogs and dogs pretreated with phenobarbital. They found a more substantial decrease in ESF in the pretreated animals suggesting the involvement of hepatic microsomal enzymes with ESF metabolism. In addition, they found a decrease in activity of plasma ESF incubated with either the 12000 g supernatant or the microsomal fraction of liver homogenate of both control and treated animals. This study confirmed the earliest work of Dukes and Goldwasser (1962), in which ESF was incubated with liver homogenate at 37°C for 30 minutes with a resulting decrease in the erythropoietic activity of ESF.

Avian Erythropoietin

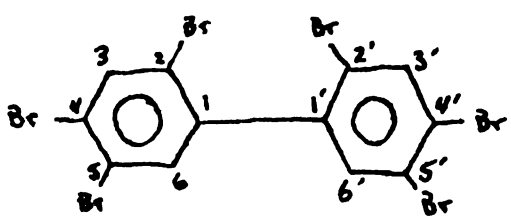
Less research has been conducted with avian erythropoietin systems than with mammalian systems. Most of the data concerning mammalian ESF has been assumed valid for avian ESF unless proven otherwise. Rosse and Waldman (1966) determined that, like mammalian ESF, avian ESF is a protein because it was inactivated by trypsin. However, avian ESF is not altered by neuraminidase as is mammalian ESF. In addition, avian ESF does not require sialic acid for biological activity. Antibody to mammalian ESF has no effect upon avian ESF. Rosse and Waldman also determined that mammalian ESF had no significant erythropoietic effect

in birds and vice versa. Mammalian ESF exhibits biological activity within all tested mammal species; avian ESF is active in all tested bird species. Even though mammalian and avian ESF have significantly different protein structure the resulting erythropoietic activity is similar.

Prior to hatching, erythropoiesis occurs in both the bone marrow and the yolk sac. After hatching, the marrow is the sole site of red cell production (Wilt, 1974; Ingram, 1974). Avian species respond to changes in O_2 content of the atmosphere and to hypoxia in the same manner as mammals. (Rosse and Waldman, 1966). Cobalt has similar effects on both avian and mammalian ESF levels (Davis et al., 1945). Because cobalt affects the kidney, it is thought that the kidney is the primary source of avian ESF. Androgens and estrogens exhibit effects in avian species similar to their effects in mammals (Nirmalin, 1972); however the mechanism is not yet known in either class of vertebrates.

Polyhalogenated Biphenyls

Polybrominated biphenyls (PBB), commercially produced as BP 6 or Firemaster FF-1, are primarily composed of isomers containing six bromine molecules per biphenyl molecule in the following configuration (Jacobs et al., 1976):



Other isomers present are tetra-, penta-, and heptabromobiphenyls,

collectively constituting 26% of the total. Other residues with varying degrees of bromination comprise the remaining 11% (Kerst, 1974). The average bromine content of Firemaster FF-1 is 75%.

PBBs are solid at room temperature with a softening point of 72°C and a decomposition temperature of 300°C. The density is 2.57 g/cc. at 26°C. They are extremely insoluble in water; 11 parts PBB dissolve per million parts water. They are soluble in various organic solvents, especially dioxane, toluene, styrene and benzene, as well as lipids and oils (Kerst, 1974). Research by Ruzo and Zabik (1974) indicates that the compound undergoes reductive dehalogenation and ring methoxylation upon irradiation with ultraviolet light with nearly 7 times the reactivity of polychlorinated biphenyls (PCB).

Although PBBs are absorbed through respiratory mucosa and the epidermis, in most studies, the chemical has been administered in food. Studies by Fries and coworkers (1975) on cows accidently contaminated by PBB showed levels in fat ranging from 205 to 2480 ppm. Average amounts in other tissues (as a percent of the amount in body fat) were as follows: milk fat 34.5, liver 3.14, kidney 2.34, lung 0.45, heart 2.07, muscle 1.97, brain 0.57, and blood 0.12. The amount of PBB in tissue is dependent on original dosage, length of time of dosage, and time elapsed from last treatment. Initially PBBs are found throughout the body, with the greatest accumulation in liver and muscle (Norris, 1975). Over time they are redistributed to the adipose tissue (Lee et al., 1975), although PBB's remain in the liver to some extent due to the chemical-metabolizing action of the organ.

PBBs are excreted primarily in the feces. Within 24 hours after initial treatment with octabromobiphenyls (OBB) Norris et al. (1975)

found 62% of the administered radioactive labeled compound eliminated in the feces. Less than 1% of the original dose was excreted through urine and through expired air. A half-life of 24 hours was exhibited for the first OBB eliminated. The half-life of the remaining compound was more than 16 days. PBBs are additionally eliminated in eggs of fowl and milk of cattle, due primarily to the high lipid content of both (Ringer and Polin, 1977; Detering et al., 1975b).

Due to their redistribution and concentration in adipose tissue, PBBs have a greater toxicity in extremely large amounts and over long periods of exposure. Dietary concentration of PBBs greater than 525 ppm causes immediate and continued refusal of that food by white leghorn chickens, leading to death by starvation. Lesser amounts, but above 125 ppm, decrease the total food intake substantially (Ringer and Polin, 1977). Experiments by Babish et al. (1975) show similar food refusal by Japanese quail: no effect below 100 ppm, total refusal above 500 ppm.

A broad effect of feeding chickens PBBs is a decrease in body weight and most organ weights (Ringer and Polin, 1977). They found reductions in size and weight of the spleen, testes and comb. However, they have found an increase in weight of the liver and thyroid (Ringer and Polin, 1977; Norris et al., 1975; Lee et al., 1975). Lee and coworkers found hypertrophy in the centrilobular hepatic cells of rats fed 100 to 1000 ppm octabromobiphenyls (OBB) for 2 weeks. They also observed an increase in the number of inclusions of osmiophilic lipid droplets within the liver cells, but found a reduction in the number of glycogen particles. Both were shown to return to normal after cessation of OBB treatment. Electron microscopy showed proliferation of smooth endoplasmic reticulum and disruption of the rough endoplasmic reticulum.

Associated with the morphological changes in the liver of Japanese quail is an increase in hepatic microsomal enzymes. Babish et al. (1975) found elevated levels of the enzymes aniline hydroxylase, aminopyrine, N-demethylase, N-methylaniline, and p-nitroanisole o-demethylase. Cecil and coworkers (1975) found that hepatic enzymes were depressed after treatment with PBB the first 24 hours, but markedly elevated after that time. They measured the induction of hepatic microsomal enzymes as a function of pentabarbital sleeping time. Drugs similar to phenobarbital increase hepatic metabolism of pentabarbital, and therefore decrease its duration of action and decrease the sleeping time of the subject. Other drugs, such as 3-methylcholanthrine have the opposite effect (Conney, 1967). In addition, an increase in aminolevulinic acid synthetase (ALAS) was observed by Strik (1973a, 1973b) in Japanese quail fed PCBs or PBBs. The enhanced ALAS activity, in conjunction with the induction of other hepatic enzymes, increased the amounts of hemoglobin precursors present, and caused porphyria in those quail.

Induction of hepatic microsomal enzymes may be a protective reaction of an animal to rid itself of deleterious compounds like PBB; however, since the liver enzymes have the potential to degrade circulating proteins as well, the result is often more harmful than beneficial. Among the circulating proteins destroyed by the liver are the hormones. This in part is reflected by changes in egg production in female chickens and in the reduction in comb size of cockerels (Ringer and Polin, 1977). There are additional reports that androgen levels are decreased in rats, mice and chickens fed PCBs (Sanders and Kirkpatrick, 1975, Nowicki et al., 1972; Platnow and Funnell, 1971). Cecil and coworkers (1975) observed that a greater amount of microsomal

enzyme induction, and thus more androgen catabolism, occurs with PBB than with PCB. Sanders and Kirkpatrick (1975) also report a decrease in glucocorticoid levels with administration of PCB in brook trout. Batomsky and Murthy (1975) found increased thyroxine (T_4) metabolism in rats treated with PCBs. They determined that the decrease in T_4 was partially due to enhanced hepatic T_4 UDP glucuronyl transferase activity (Batomsky et al., 1976). Treatment with PCB's also resulted in increased biliary excretion of T_4, possibly due to displacement of T_4 from serum binding proteins. Ringer and Polin (1977) demonstrated thyroid hyperplasia in chickens, also.

Although moderate amounts of PBBs have been found within the kidneys, it is unlikely that there is much damage to the renal tissue. Iwamoto (1973) analyzed the urine of mink fed PCBs and found all tests indicate absence of kidney damage in the animals. Urine specific gravity and levels of protein, glucose, ketones and blood were all within accepted values for mink. He also determined that the blood urea nitrogen (BUN) levels were normal. Norris and coworkers (1975) found increased kidney weight in rats fed OBB, in addition to the presence of hyaline degenerative cytoplasmic lesions in some animals. Urinalysis, though, showed no changes in pH or in glucose, protein, ketone or blood levels of the treated rats when compared with control animals. Hansell and Ecobichon (1974) studied the effects of PCBs on rat kidney. They found changes in renal tubule cells including increase in the number of vacuoles, lipid droplets, microbodies, lysosomes, smooth endoplasmic reticulum, as well as alterations in mitochondria of some tubule cells. They failed to find any damage to the renal glomerulus.

In 1972, Rehfeld et al. reported pronounced changes in red cell numbers and hemoglobin concentration of chickens fed different levels of PCBs. Iturri and coworkers (1974) observed similar results in cockerels, along with changes in other cardiovascular parameters. Heineman (1976) found decreased hemoglobin and hematocrit levels in cockerels fed PBBs, although the effects required greater amounts of PBB and longer feed administration than similar studies with PCBs. Ringer and Lack (unpublished) observed changes in bone marrow red cell composition in cockerels fed 200 ppm PBB. They found fewer erythroblasts, early and mid- polychromatic erythrocytes, and mature erythrocytes in bone marrow smears prepared from the PBB-treated birds compared to similarly prepared smears from control birds.

OBJECTIVES

1. To determine changes in hematocrits of cockerels fed 150 ppm PBB.

2. To determine changes in hemoglobin concentration of cockerels fed 150 ppm PBB.

3. To determine whether or not changes in hematocrits and hemoglobin concentration in cockerels fed 150 ppm PBB are due to altered levels of plasma erythropoietin (ESF).

MATERIALS AND METHODS

Animals

In the first experiment a total of twenty-seven Single Comb White Leghorn (SCWL) cockerels were obtained from Rainbow Trail Hatchery (St. Louis, Michigan). They were divided into three groups: *ad libitum* control, control pair-fed to 150 ppm, and treated at 150 ppm PBB. The chicks were assigned to groups so that the means of initial weights of all groups were equal. They were housed in standard chick starting batteries until six weeks of age; at this point they were transferred to growing batteries until termination of the experiment. Fifty one-week old SCWL cockerels were used in the second experiment. They were assigned to either the control pair-fed to 150 ppm group or treatment with 150 ppm PBB group as in the first experiment, and were housed in the same manner.

Japanese quail (*Coturnix japonica*) were used as the assay animals for the erythropoietin bioassay. In the first experiment, fifty adult males of unknown age were obtained from the Michigan State University Poultry Farm. Sixty young adult males five to seven weeks old were used in the second experiment. In both experiments, quail were housed in standard chick starting batteries. They were fed standard quail breeder QB-72 (King Milling Company, Lowell, Mich.) *ad libitum* (Table 1.).

Table 1. Quail Breeder QB-72 (King Milling Company, Lowell, Michigan)

Ingredients	Grams/Kilogram
Corn	450.2
Soybean meal, 49%	327.0
Fish meal	0.0
Meat scrap, 50%	50.0
Dehydrated alfalfa meal	45.0
Stabilized animal fat	57.0
Limestone	50.0
Dicalcium phosphate	7.0
Choline chloride, 50%	3.0
Methionine hydroxy analogue	1.0
Iodized salt	3.8
Mineral mix A.	3.0
Vitamin mix A.	3.0
Antioxidant	56.8

Feeding of SCWL Cockerels

In the first experiment, the control group was fed finely ground chick starter, CS-75 (King Milling Company, Lowell, Mich.) ad libitum (Table 2.). In both experiments the PBB treated group was fed, ad libitum, a mixture of finely ground Fire Master FF-1 (originally obtained from Michigan Chemical Co., Chicago, Ill.) and ground chick starter in a ratio of 150 parts of Fire Master FF-1 in one million parts chick starter (Appendix I: Determination of PBB Rations). An additional control group, pair-fed to 150 ppm PBB, was used to eliminate any possible effects due to any decrease in feed consumption. This group was fed an amount of chick starter equal to the amount of 150 ppm PBB ration consumed by the treated group over the previous feeding interval (generally two days). (Appendix II: Sample Pair-feeding Calculations). All groups were given a free choice of amount of fresh water daily.

Hematological Preparation

In the first experiment, packed red cell volume (hematocrit value) and hemoglobin concentration were measured in all birds after 4½ and 8 weeks of the study. In the second experiment, packed cell volume and hemoglobin concentration were measured in all surviving birds after 8 weeks of treatment.

Hematocrits were determined by drawing blood by venipuncture from each SCWL cockerel with collection into a heparinized capillary tube. These tubes were centrifuged at 4,500 rpm for 5 minutes in an International Microcapillary Centrifuge (International Equipment Co.,

Table 2. Chick Starter CS- 75 (King Milling Company, Lowell, Michigan)

Ingredients	Grams/Kilogram
Corn	621.5
Soybean meal,49%	205.0
Alfalfa, 17%	25.0
Meat and bone meal	30.0
Fish meal, 60%	25.0
Dried whey	20.0
Oats	50.0
Salt	2.5
Ground limestone	7.5
Dicalcium phosphate	7.5
Vitamin and mineral premix (5003)	5.0
Additives	1.0

Boston, Mass.). The packed red cell volume in each tube was measured using a microcapillary reader.

Hemoglobin concentration was determined by the cyanmethemoglobin method. Twenty microliters of blood from each bird was collected in a capillary tube and expelled into a spectrophotometer tube containing 5 ml of Drabkin's Reagent (Appendix III: Preparation Of Drabkin's Reagent). Each tube was agitated to aid in the lysis of the red cells to release the hemoglobin. After allowing the tubes to set for 20 minutes, the percent absorbance of each sample was measured in a Spectronic 20 Calorimeter-Spectrophotometer (Bausch and Lomb, Rochester, N.Y.) at 540 nm, against a reagent zeroing blank. The hemoglobin concentration was determined by the comparison of each sample percent absorbance against the percent absorbance of standards with known hemoglobin concentration (Appendix IV: Determination of Hemoglobin Concentration).

Erythropoietin Assay

The primary purpose of this research was to compare the ESF levels in control and PBB treated SCWL cockerels. The ESF bioassay was adapted from Rosse and Waldman (1965). After 8 weeks of feeding, the birds in all groups were sacrificed and bled by decapitation. Each blood sample was collected in a 10 ml, plastic centrifuge tube and was allowed to clot. The samples were centrifuged in a Sorval Superspeed RC 2-B (Sorval, Inc., Norwalk, Conn.) at 20,000 x g at 4^{o}C for 20 minutes. The supernatant serum was collected and frozen until assayed.

Previous research (Gordon and Weintraub, 1962) has shown that the sensitivity of the ESF assay can be increased by pretreatment of the assay animals with cobalt, hypoxia, transfusion or other methods that

increase the red cell count of the animals. The most successful method exposes the animals to hypoxia in the form of decreased atmospheric pressure for a period of time. In the first experiment, the quail were exposed to 456 torrs, 17 hours per day for 21 days. In the second experiment, the birds were exposed to 540 torrs, 14 hours per day for 20 days and 16 hours per day for the last ten days (for a total of 30 days). In each experiment, the quail were gradually acclimated to the decreased pressure over a period of 3 to 4 days. The decreased atmospheric pressure was achieved by drawing a vacuum through a potentially air-tight chamber. The vacuum itself and an inlet valve were adjusted to provide partial pressure and to provide a continuous influx of fresh air.

The result of such treatment was polycythemia in the quail and subsequent reduction of the birds' own ESF production. Exogenous ESF, in the form of chicken serum, was injected into each quail to determine the erythropoietic activity of the serum sample. In the first experiment, each quail was injected intraperitoneally with 0.5 ml of a sample of either control, pair-fed control, or PBB treated chicken serum on the first and second day after exposure to hypoxia. The next two days each bird was injected with 1.0 ml of the same serum sample. In the second experiment, each quail was injected intraperitoneally with 1.0 ml of a serum sample for 3 days after hypoxia pretreatment. During the serum injection period in both experiments, the birds were watered daily but were not fed. Starvation increases the sensitivity of the assay by depleting the quails' stores of thymidine, forcing them to incorporate primarily radioactive thymidine into the newly-produced erythrocytes.

On the day following the last injection of chicken serum, 0.5 microcuries of thymidine 2-C^{14} in 0.5 ml 0.15 M NaCl was injected intraperitoneally into each quail. In the second experiment, a third group of quail was injected with only thymidine at the same time as the quail injected with pair-fed control and PBB treated chicken serum. This group represents a further control indicating the basal erythropoietic activity of hypoxic-polycythemic quail. Four days after the thymidine 2-C^{14} injections, the quail were sacrificed and bled by decapitation. The blood samples were collected in 10 ml plastic centrifuge tubes containing 2 drops of heparin to prevent clotting. These samples were then prepared for scintillation counting.

Scintillation counting of whole blood samples is somewhat difficult due to the color quenching effects of the blood (Appendix V: Scintillation Counting). This problem was overcome by removing the hemoglobin from the erythrocytes, leaving nucleated erythrocyte ghosts (Harris and Brown, 1971). The ghosts were prepared by centrifuging the heparinized whole blood samples at 2,000 x g for 10 minutes in a Sorval Superspeed RC 2-B. The serum was discarded and the packed red cells were washed twice in 1.0% NaCl at $4^{\circ}C$ with centrifugation at 2,000 x g for 10 minutes. The erythrocytes were hemolyzed in 10 volumes (roughly 10 ml) of a solution of 10 uM Tris HCl (pH 7.4) and 4 mM $MgCl_2$ and centrifuged at $4^{\circ}C$ at 20,000 x g for 10 minutes. Each sample was washed 8 times in 5 volumes of the previous solution, alternately with and without 0.3 M sucrose, and centrifuged at 20,000 x g for 10 minutes (Appendix VI: Erythrocyte Ghost Preparation Flow Chart). The ghosts were not resuspended after last centrifugation to provide a more concentrated preparation.

To prepare the nucleated erythrocyte ghosts for counting, 140 mg of the ghosts from each sample was placed into a counting vial. To this amount, 1.4 ml of Unisol Tissue Solubilizer (Isolab, Akron, Ohio) was added. After agitation the vials were allowed to stand at room temperature overnight (approximately 14 hours) or until no protein fibers remained. 14 ml of Unisol Complement Scintillation Fluid (Isolab, Inc., Akron, Ohio) was added to each vial. The vials were again agitated until the solubilized proteins were completely in solution. All samples were counted on a Beckman LS 100 C Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, California). The data obtained were converted from counts per minute to degradations per minute prior to statistical analysis (Appendix V: Scintillation Counting).

Statistical Analysis

All data were analyzed by one-way analysis of variance. Since the first experiment involved 2 similar control groups against 1 treated group, multiple comparisons among group means were made using designed orthagonal contrasts (Gill, unpublished). The second study involved 1 or 2 dissimilar control groups against 1 treated group; therefore, multiple comparisons among group means were made using the Bonferroni t-statistics, a non-orthagonal designed contrast (Gill, unpublished).

RESULTS

Mortality

After 4½ weeks of feeding in the first experiment, there was a loss of 1 SCWL cockerel from the PBB-treated group. This represented a mortality rate of 11%. The ad libitum and pair-fed control groups exhibited no change in numbers of birds after 4½ weeks. At the end of the first experiment, a total of 4 of the original 9 cockerels had died, representing a mortality rate of 44% of the PBB-treated group. The ad libitum control group showed a loss of 2 birds, a mortality of 22%. The pair-fed control group exhibited no deaths even after 8 weeks of treatment.

In the second experiment, only 1 SCWL cockerel out of the original 25 in the pair-fed control group died over the course of the experiment. The mortality rate after the full 8 weeks was 4%. After 8 weeks, 7 of the 25 birds in the PBB fed group died, a mortality rate of 28%.

Hematocrit

The mean hematocrit values from the first experiment at 4½ and 8 weeks and the second experiment at 8 weeks were compiled in Table 3. After 4½ weeks of feeding in the first study, a decreased mean hematocrit of 26.4% was observed in the group of SCWL cockerels fed 150 ppm PBB. The mean values for the ad libitum and pair-fed control groups were 28.4% and 29.4%, respectively. The average of these values

Table 3. The effect of feeding rations containing 0 and 150 ppm PBB (as Fire Master FF-1) on hematocrit, and hemoglobin concentration of SCWL cockerels.

Parameter	Dietary Treatment[a]		
	Ad libitum control	Pair-fed control	150 ppm PBB
Hematocrit (%)			
First 4½ weeks Experiment	28.4 ± 0.5 $_{m}$ (9)	29.4 ± 0.8 $_{m}$ (9)	26.4 ± 0.9 $_{n}$ (8)[bc]
First 8 weeks Experiment	33.8 ± 0.6 $_{x}$ (7)	32.8 ± 0.9 $_{x}$ (9)	23.4 ± 0.9 $_{y}$ (5)
Second 8 weeks Experiment	----------	32.3 ± 0.6 $_{r}$(21)	24.0 ± 0.4 $_{s}$(18)
Hemoglobin (g/100 ml)			
First 4½ weeks Experiment	9.8 ± 0.2 $_{m}$ (9)	9.9 ± 0.2 $_{m}$ (9)	8.8 ± 0.2 $_{n}$ (8)
First 8 weeks Experiment	9.8 ± 0.2 $_{x}$ (7)	10.1 ± 0.2 $_{x}$ (9)	7.2 ± 0.2 $_{y}$ (5)
Second 8 weeks Experiment	---------	9.5 ± 0.2 $_{r}$(21)	7.4 ± 0.1 $_{s}$(18)

[a] Rations fed for 8 weeks

[b] Data reported as Group mean ± Standard error (number of birds per group)

[c] Means having different subscripts are significantly different; m,n P = 0.025
r,s P = 0.01
x,y P = 0.001

were 8.6% greater than the PBB mean hematocrit. The mean hematocrit of the PBB treated group was significantly different from that of both control groups (P= 0.025). Statistically, there was no significant difference between the 2 control groups. The mean hematocrit values were computed from the hematocrits of all surviving birds in each group.

At the termination of the same study, after 8 weeks of treatment, the trends were the same as were observed after 4½ weeks. The ad libitum and pair-fed control mean hematocrits increased to 33.8% and 32.8%, respectively. The PBB group mean further decreased to 23.4%, nearly 30% lower than the control values. There was no significant difference between the mean hematocrits of the ad libitum and pair-fed control groups. The statistical significant difference between the mean hematocrit values of the control and the PBB-fed groups increased to P= 0.001. Again, the mean hematocrits of the 3 groups were obtained from the values of all surviving birds.

The results of the second study were similar to those of the first study. After 8 weeks of feeding, the mean hematocrit of the PBB fed group was 24%. The mean value of the pair-fed control group was 32.3%, 26% greater than the PBB group mean. There was a significant difference between the means of the 2 groups (P= 0.01). The mean hematocrits were calculated from those of all of the surviving PBB fed cockerels and 21 of the 24 surviving birds in the pair-fed group.

The mean hematocrits and their standard errors from both experiments are graphically portrayed in Figure 3. The increase in mean hematocrits of the control groups from 4½ to 8 weeks is evident. The decrease in means of the PBB group over the same time is also evident.

Figure 4. The effect of feeding rations of 0 and 150 ppm PBB (as Fire Master FF-1) on the mean hemoglobin concentration of SCWL cockerels. The number of birds per group is indicated in patenthesis and the standard error is designated by the vertical lines.

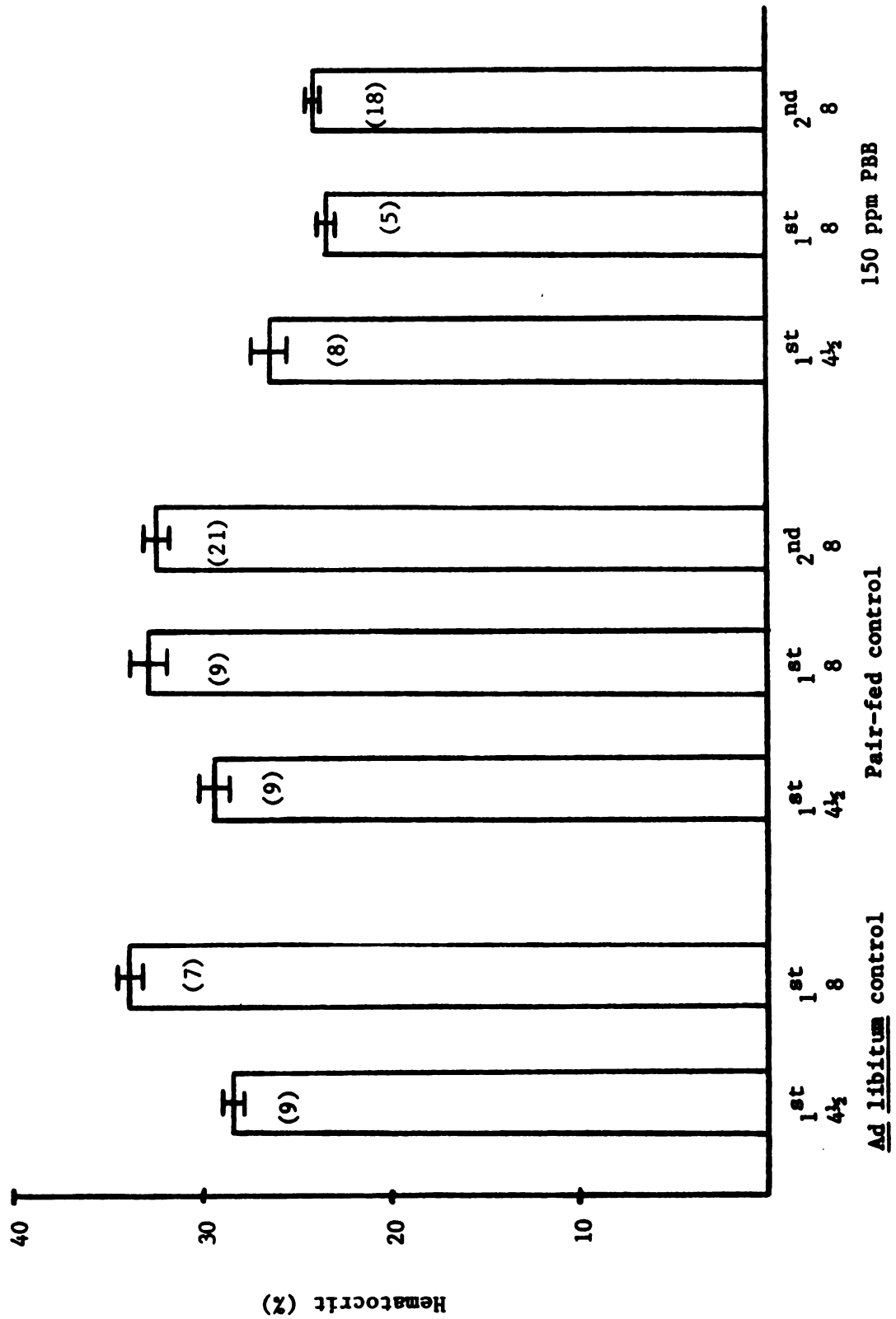

Hematocrit (%)
40
30
20
10
(9)
(7)
(9)
(9)
(21)
(8)
(5)
(18)
1st 4½
1st 8
1st 4½
1st 8
2nd 8
1st 4½
1st 8
2nd 8
Ad libitum control
Pair-fed control
150 ppm PBB

Figure 3.

The graph additionally shows that there is little difference between the first and second experiments after 8 weeks of treatment.

Hemoglobin Concentration

Table 3 contains the mean hemoglobin concentrations from both experiments. In the first study, the mean hemoglobin concentration for the PBB treated cockerels was 8.8 g/100 ml. This value was nearly 11% lower than the ad libitum and pair-fed control group means, 9.8 g/100 ml and 9.9 g/100 ml, respectively. After 4½ weeks, a significant difference was found between the PBB fed group and the 2 control groups (P= 0.025). There was no statistical difference between the means of the control groups. The mean hemoglobin concentrations were determined from the values of all surviving birds in each group.

After 8 weeks of the first study, there was a greater difference between the mean hemoglobin concentrations of the PBB treated group and the 2 control groups (P= 0.001). The mean hemoglobin concentrations of the ad libitum and pair-fed control groups were consistent with the values at 4½ weeks to 9.8 g/100 ml and 10.1 g/100 ml, respectively. The mean of the PBB fed group decreased to 7.2 g/100 ml, 26% lower than the control means. Again, the mean hemoglobin concentrations were determined from the values of all surviving birds within each group.

The results of the second experiment were similar to those of the first study at 8 weeks. After 8 weeks of treatment, the mean hemoglobin concentration of the PBB fed group was 7.4 g/100 ml, 22% lower than the pair-fed control mean of 9.5 g/100 ml. The PBB mean hemoglobin concentration was significantly different from the control mean at P= 0.01. The means were derived from the values of the 18 surviving

Figure 3. The effect of feeding rations of 0 and 150 ppm PBB (as Fire Master FF-1) on the mean hematocrit values of SCWL cockerels. The number of birds per group is indicated in parenthesis and the standard error is designated by the vertical lines.

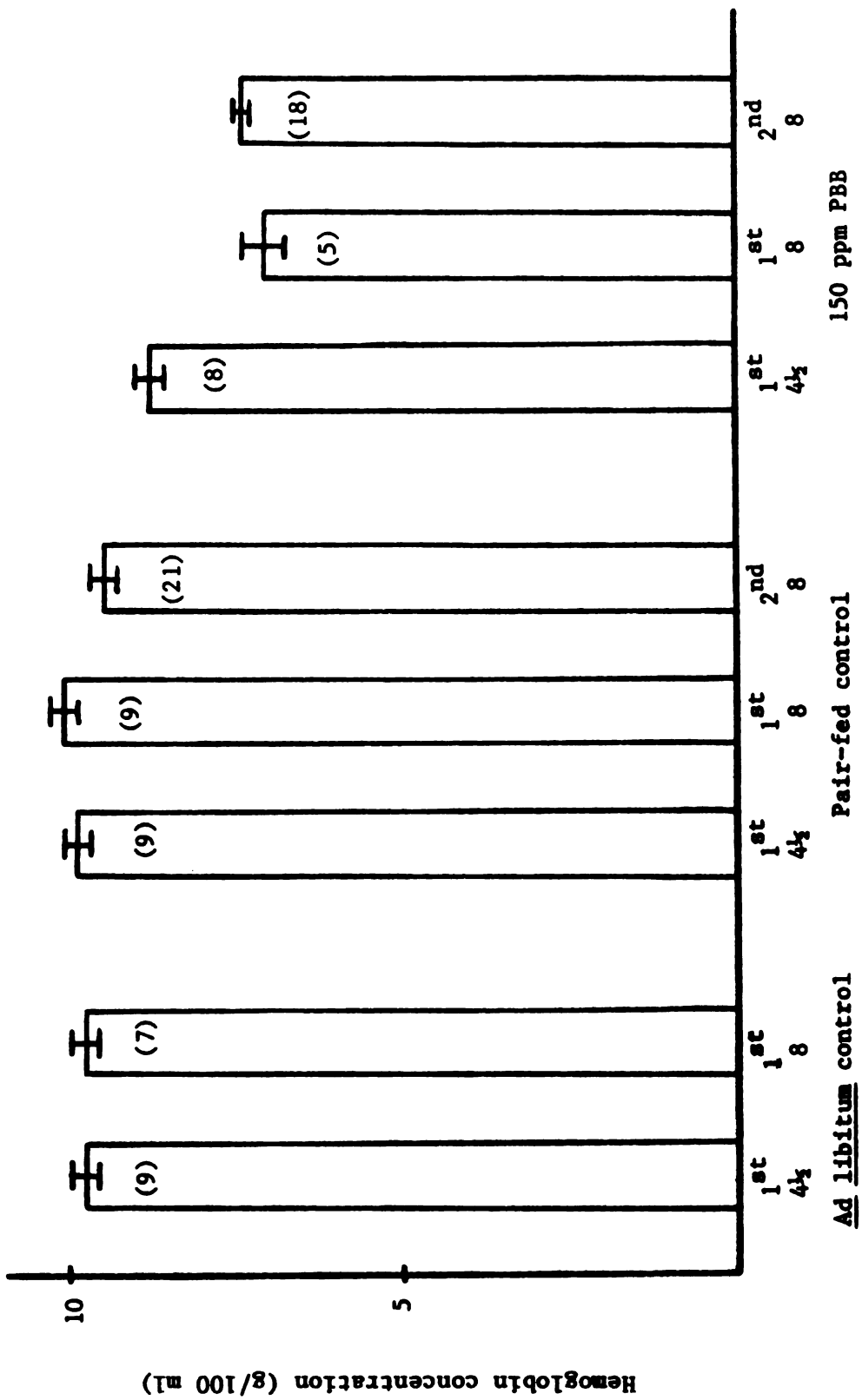

Hemoglobin concentration (g/100 ml)
10
5
(9)
(7)
(9)
(9)
(21)
(8)
(5)
(18)
1st 4½
1st 8
1st 4½
1st 8
2nd 8
1st 4½
1st 8
2nd 8
Ad libitum control
Pair-fed control
150 ppm PBB

Figure 4.

cockerels in the PBB group and from 21 of the 24 surviving pair-fed cockerels.

Figure 4 represents the mean hemoglobin concentrations of the first experiment at 4½ and 8 weeks, and the second experiment at 8 weeks. The graph shows that the means remained constant in the ad libitum and pair-fed control groups from 4½ to 8 weeks. In contrast, the means of the PBB treated cockerels decreased over the same time period. Additionally, the graph shows that there was little difference in the mean hemoglobin concentrations of the PBB treated and pair-fed control groups at 8 weeks of treatment between the first and the second experiments.

Erythropoietin Levels

The mean amounts of thymidine 2-C^{14} incorporated into quail erythrocytes for various treatment groups are compiled in Table 4. Determination of ESF levels at 4½ weeks was not made in the first study since it was not possible to collect enough blood from the chicks to prepare 3 milliliters of serum. In the first study, after 8 weeks of treatment, there was no significant change in the mean amount of thymidine 2-C^{14} incorporated into quail erythrocytes as a result of injection of serum from the cockerels of any of the 3 groups. The mean incorporation, in terms of degradations per minute per 140 mg packed RBC ghosts (DPM), were 3954.5, 3513.0, and 3239.9, respectively for the ad libitum control, the pair-fed control, and the PBB treated groups. These values indicated that there was no difference in the ESF levels among the 3 groups. The ad libitum control group mean was determined from 4 of the 7 surviving cockerels in that group. Of the remaining 3

Table 4. The effect of injection of either no serum or serum from SCWL cockerels fed rations of 0 and 150 ppm PBB (as Fire Master FF-1) on quail erythrocyte thymidine 2-C^{14} incorporation.

Parameter	Serum Treatment			
	No serum	Ad libitum control serum	Pair-fed control serum	150 ppm PBB serum
Thymidine 2-C^{14} incorporation (DPM per 140 mg packed RBC ghosts)				
First experiment 8 weeks	----------	3954.5±343.2 (4)	3513.0±799.0 (8)	3239.9±1267.9 (3)[a]
Second experiment 8 weeks	1397.8±177.5$_x$(8)	----------	1640.7±105.8$_y$(19)	1028.3±77.7$_z$ (17)

[a]Data reported as Group means ± Standard error (number of birds per group)

Means having different subscripts are significantly different; P = 0.01

survivors, 1 did not provide enough serum to assay. The DPM of the other 2 survivors were statistically excluded as residuals because their values were extremely high (8510.2 and 7817.7 DPM) when compared to the mean (3954.5 DPM). The pair-fed control mean was calculated from 8 of the 9 surviving birds. One bird lacked a sufficient blood sample to assay. The mean for the PBB fed group was determined from the values of 3 of the 5 birds that survived treatment. The remaining 2 values were not obtained since those quail died after injection with the prepared serum.

In the second study, after 8 weeks of treatment, the amounts of thymidine 2-C^{14} incorporated into the quail erythrocytes, as reflected by the mean number of degradations, were 1397.8 DPM, 1640.7 DPM, and 1028.3 DPM for the quail not injected with any serum, the quail injected with control serum, and the quail injected with PBB serum. These values were all significantly different from one another at P= 0.01. The mean thymidine 2-C^{14} incorporation in quail not injected with any serum was 15% less than that in quail injected with serum from pair-fed control cockerels, and 26% greater than that in quail injected with serum from PBB fed cockerels. There was a 37% difference in the mean thymidine 2-C^{14} incorporation in quail injected with serum prepared from pair-fed control cockerels and from PBB fed cockerels. Serum from only 19 of the 21 cockerels that survived 8 weeks of treatment was used to determine the mean of the pair-fed cockerels. Sufficient serum was not obtained from 1 bird, and 1 quail died after injection of the serum. The PBB group mean was determined from the results of all 18 surviving birds. Again, though, 1 value was statistically excluded as a residual.

Figure 5 graphically portrays the mean erythrocyte thymidine 2-C^{14} incorporation in quail injected with the various sera. The values from the first experiment were not directly comparable to those of the second experiment due to differences in the procedures of the 2 studies. In the first experiment, even though the mean DPM decreased from the ad libitum control group through the PBB treated group, the extent of the maximum range was roughly equal over all 3 groups. In the second experiment, the differences among the 3 groups are evident.

Figure 5. The effect of injection of either no serum or serum prepared from SCWL cockerels fed 0 and 150 ppm PBB (as Fire Master FF-1) on the mean quail erythrocyte thymidine 2-C^{14} incorporation. The number of birds per group is indicated in parenthesis and the standard error is designated by vertical lines.

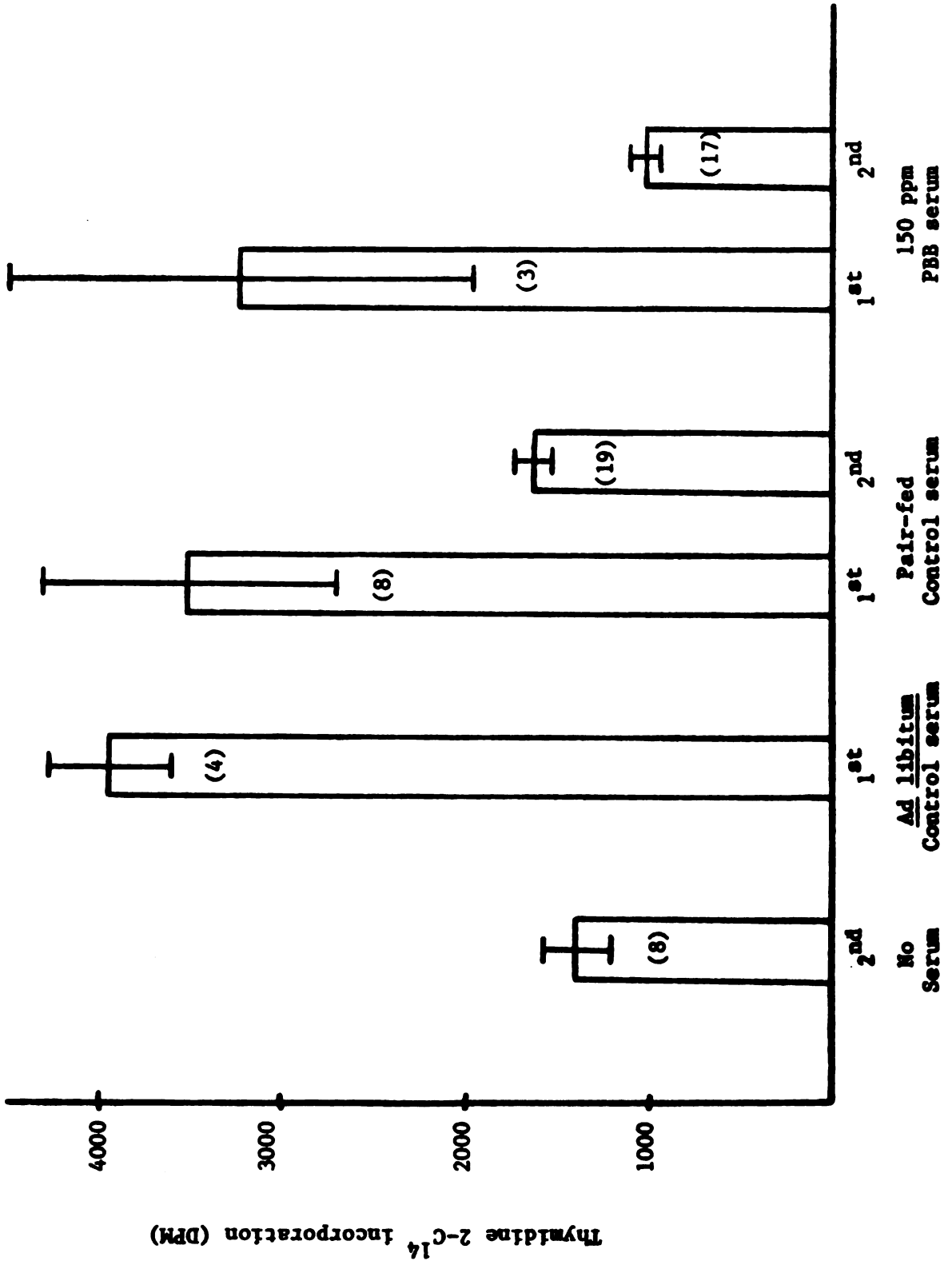

Thymidine 2-C14 incorporation (DPM)
4000
3000
2000
1000
(8)
(4)
(8)
(19)
(3)
(17)
2nd
1st
2nd
1st
2nd
1st
2nd
No Serum
Ad libitum Control serum
Pair-fed Control serum
150 ppm PBB serum

Figure 5.

DISCUSSION

Anemia is defined as a decrease in either the ratio of red blood cells to plasma, the size of the red cells, or the amount of hemoglobin contained in the red cells. It is unlikely that the decrease in packed cell volume (hematocrit) and hemoglobin concentration was due to increased plasma volume since similarly treated birds were found to have generalized edema and decreased body weight (Heineman, 1976).[1] The decreased hematocrits and hemoglobin concentrations of PBB fed cockerels were also not due to decreased feed intake. There was no difference in hematological values between the ad libitum and pair-fed control birds in the first study. Because of this it was decided that the ad libitum control group was no longer a necessary group; thus, the second experiment consisted of only pair-fed control and PBB treated groups. The first and second studies showed a definite decrease in the hematocrits and hemoglobin concentrations of the cockerels fed 150 ppm PBB for 8 weeks as compared to those of the control birds. Thus, PBB administration, at 150 ppm, results in anemia in SCWL cockerels.

The mean erythrocyte thymidine 2-C^{14} incorporation data obtained from the first experiment were not extremely accurate due to the small number of birds in each group and the large standard error for each

1. Additional data concerning the effects of PBB and body weight, mortality and food consumption can be found in the M.S. thesis of Fredrick W. Heineman, MSU, 1976.

group mean. Each standard error was greatly reduced in the second experiment, so the data were considered more reliable. The amount of thymidine 2-C^{14} incorporated into the quail erythrocytes indicated the relative level of ESF present in each group of serum. The exogenous ESF injected into each quail stimulated its erythropoietic system to produce additional erythrocytes. As the red cells were produced, the thymidine 2-C^{14} injected was incorporated into the cells. Due to the lack of an avian ESF standard no direct numerical correlation could be made between thymidine 2-C^{14} DPM and the amount of ESF injected, higher mean DPM indicated greater levels of ESF and lower mean DPM indicated lower levels of ESF. It was assumed that the mean DPM value of the group of quail not injected with any serum represented the basal erythropoietic activity of the hypoxicpolycythemic quail, activity due only to endogenous ESF. Quail erythropoietic activity was increased by the addition of serum prepared from pair-fed control cockerels, presumably due to ESF present in the serum. The amount of ESF present in the serum of the pair-fed control birds reflected the amount of ESF necessary to keep the animals in erythropoietic homeostasis. Erythropoietic activity was decreased in the quail injected with serum from the PBB fed cockerels. The decrease in erythropoietic activity of the test animals indicated that the ESF level of the PBB birds was lower than that of the birds not fed PBB. In addition, since the erythropoietic activity of the quail injected with serum from PBB fed cockerels was lower than the basal quail erythropoietic activity, there was a possibility that a component in the serum inhibited the endogenous quail ESF. Similarly, this component would decrease the activity of the

erythropoietic system in the PBB fed cockerels, creating the observed anemia.

In general, there are many mechanisms through which anemia may occur. These include the following factors: 1) decreased endogenous hormone levels, 2) altered bone marrow erythroblasts, 3) decreased life span of the red cells leading to increased hemolysis, 4) inability of the renal oxygen sensor to detect changes in blood oxygen, 5) insufficient renal ESF production, 6) increased ESF inhibition, and 7) increased hepatic ESF metabolism.

It is unlikely that the first three factors were the primary cause of PBB-induced anemia, singly or in combination with one another. Due to the nature of the erythropoietic negative feedback system, any reduction in the number of red cells stimulates increased ESF formation (Rosse and Waldman, 1966). In contrast administration of 150 ppm PBB resulted in decreased ESF levels. Since endogenous levels of hormones such as androgens, glucocorticoids and thyroxine have been found to be lower with administration of PBB, decreased hormone levels may have an indirect effect upon the erythropoietic system. This mechanism of PCB action has been postulated by Rehfeld et al (1971) and Platonow and Funnell (1971) although neither assayed for ESF activity. Decreased hormone levels may initiate or contribute to PBB-induced anemia by removing some of the stimuli that result in increased red cell formation during growth. The degree to which these three decreased hormones affect erythropoiesis in PBB-treated animals can be determined by replacing the endogenous hormones with exogenous hormones in the diet. Even though fewer erythroblasts, and the cells into which they differentiate, were observed in bone marrow smears from PBB-treated

cockerels (Ringer and Lack, unpublished), this does not necessarily indicate that the erythropoietin responsive stem cells failed to respond to ESF, or that they failed to differentiate into erythroblasts after ESF stimulation. If this had occurred the negative feedback system would have increased ESF production. Rather, the bone marrow observation could additionally indicate absence of plasma ESF, and therefore absence of maturing red cells. This could be determined by injecting large amounts of ESF into the PBB treated animals. If the bone marrow were indeed damaged, there would not be any change in the hematological values in the animals. Finally, the life span of red cells could have been decreased in the PBB treated cockerels. Strik (1973b) found that PBB-fed quail exhibited porphyria and increased ALAS levels. Although porphyric animals often exhibit hemolytic anemia (Merck, 1966), the porphyria is usually a symptom caused by the increased levels of hemoglobin precursors, including ALAS, rather than the result of the anemia. The actual life span of red cells in PBB-treated animals can be determined by labeling newly-produced red cells with a radioactive isotope, sampling the blood at specific intervals and testing for radioactivity, and calculating the length of time required for the majority of the label to disappear (Rodnan, 1957). The normal life span of fowl erythrocytes is 25 to 30 days (Bell and Freeman, 1971). The radioactive label would disappear before day 25 if PBB increased the rate of red cell destruction and decreased the life span of the average red cell. Since these three mechanisms, if altered, would have increased the plasma concentrations of ESF, it was improbable that the PBB-induced anemia was due primarily to decreased hormone levels, altered bone marrow, and/or decreased red cell life span.

While faulty renal oxygen sensors, insufficient renal ESF production, increased ESF inhibition and increased ESF metabolism should all result in decreased plasma ESF levels. Neither Iwamoto (1973) nor Norris et al. (1975) found perceptible changes in renal filtration function with either PCB or PBB treatment. In addition, there have not been any reports of damage to the renal glomerulus, the site of renal ESF production (Fisher et al., 1965) and possible site of the renal oxygen sensor. Also due to the decrease in cell numbers in the PBB-treated animals, one would expect additional incorporation of hemoglobin into those few cells produced. Strik (1973b) reported that PBB's increased ALAS, a precursor in hemoglobin formation, but the majority of this compound did not seem to be incorporated into red cells, a condition which resulted in porphyria. Also, Iturri et al. (1974) showed that the mean corpuscular hemoglobin concentration (MCHC) was not significantly changed after PCB treatment. Rather, there was a trend toward decreased MCHC with administration of increasingly greater amounts of PCB. This indicated that there was a lack of active plasma ESF. Further research is required to determine whether or not decreased plasma ESF levels with administration of 150 ppm PBB were due to failure of the renal oxygen sensor, or the inhibition of ESF synthesis. Inoperative renal oxygen sensors can be detected by exposing the PBB-treated animals to hypo- and hyperoxic environments. If, indeed, the sensors were defective the hematological values of the hypo- and hyperoxic animals would be similar to those of the control animals. Failure of the renal glomerular epithelial cells to produce ESF can be determined by measuring the levels of adenyl cyclase, cyclic-AMP and ESF present in the kidneys of PBB-fed animals. Production of ESF in the

glomerulus, or lack of it, can also be determined by the use of a fluorescent ESF antibody (Fisher et al., 1975).

According to research done to date, the most probable cause for the PBB-induced anemia was inactivation of large amounts of ESF. The ESF could have been inactivated by an ESF inhibitor or by hepatic degradation. Initially, active ESF may have been displaced from its protective serum binding proteins, as occurs with thyroxine (Batomsky, 1974). This would render the hormone more susceptible to degradation by the liver microsomal enzymes. Many other hormones are destroyed in this manner, among them androgens, glucocorticoids, and thyroxine. Since ESF is degraded in the liver by hepatic microsomal enzymes (Roh and Fischer, 1971) and PHB's induce hepatic microsomal enzymes (Conney, 1967) it follows that PHB's should increase ESF metabolism by increased hepatic microsomal enzymes. This hypothesis can be tested by Roh and Fisher's perfusion techniques. Plasma containing high concentrations of ESF can be perfused through livers of animals pretreated with PBB. In addition, homogenates of liver from animals exposed to PBB can be incubated with plasma containing concentrated ESF. If ESF metabolism were indeed increased with administration of PBB, the plasma from the perfusion and homogenate experiments would have little erythropoietic activity.

The decrease in ESF in the PBB-treated cockerels could have additionally been due to inhibition of plasma ESF by some component. This hypothesis is strongly supported by the limited data from the final experiment. Unfortunately, little is known concerning ESF inhibitors. Dukes et al. (1972, 1975) determined that certain prostaglandins potentiate the erythropoietic activity of ESF and are possibly a cofactor necessary for ESF bioactivity. They proposed that the ESF

inhibitor, a protein, has a higher affinity for the prostaglandin cofactor than normal ESF does. Erslev et al. (1971) found an inhibitor that was a lipid. They thought it acted by displacing the prostaglandins normally bound to ESF. This would inactivate the ESF and possibly make it more susceptible to degradation by the liver. The presence of PBB could have increased the amount of inhibitor produced and thus could have decreased erythropoiesis in the cockerels with the resultant anemia. There have not been any reports on the effects of PCBs or PBBs on prostaglandins in living systems. The existence of an ESF inhibitor could be determined by adding active ESF to plasma from PBB-treated animals. Up to a certain point, the preparation would have little or no activity. At that point, sufficient ESF would exist to bind to all of the inhibitor molecules. Past that point, the erythropoietic activity of the preparation would increase as ESF was added.

SUMMARY

1. The hematocrits were significantly decreased in the first experiment cockerels fed 150 ppm PBB for 8 weeks relative to the ad libitum and pair-fed controls. Therefore, the change in hematocrits was directly attributed to PBB.

2. Hemoglobin concentrations were significantly decreased in the first experiment cockerels fed 150 ppm PBB for 8 weeks relative to the ad libitum and pair-fed controls. Therefore, the change in hemoglobin concentration was directly attributed to PBB.

3. In the second experiment, plasma erythropoietin (ESF) levels were significantly decreased in the cockerels fed 150 ppm PBB for 8 weeks relative to the pair-fed controls. Therefore, the change in plasma ESF levels was attributed to PBB.

APPENDICES

APPENDIX I

PBB RATION PREPARATION

A premix of 1% PBB was prepared by adding 30 g. of pulverized Fire Master FF-1 which had been passed through a sieve (U.S. Standard #30) to 2970 g. finely ground, similarily sieved chick starter CS-75 (King Milling Company, Lowell, Michigan). The final 150 ppm PBB ration was prepared in 4 kilogram quantities by combining 60 g. 1% premix with 3940 g. ground, unsieved chick starter CS-75. All ration mixing was done on a Paul G. Abbé, Inc., feed mixer (Little Falls, N.J.), tumbling for 15 minutes in 30 pound capacity feed cans.

APPENDIX II

SAMPLE PAIR-FEEDING CALCULATIONS

Total PBB ration given = G_{PBB}	(2500 g)
Total PBB ration not consumed = R_{PBB}	(300 g)
Total PBB ration consumed = $C_{PBB} = G_{PBB} - R_{PBB}$	(2500 g - 300 g = 2200 g)
Number of PBB fed birds = B_{PBB}	(22)
PBB ration per bird = C_{PBB}/B_{PBB}	(2200 g/ 22 = 100 g)

The amount of PBB ration consumed per bird every 2 days is equal to the amount of chick starter that is to be fed to the pair-fed control birds over the next 2 days.

Chick starter per bird = PBB ration per bird	(100 g = 100 g)
Number of pair-fed birds = B_{PF}	(24)
Total chick starter given = G_{PF}	(unknown)
G_{PF} = Chick starter per bird x B_{PF}	(2400 g = 100 g x 24)

APPENDIX III

PREPARATION OF DRABKIN'S REAGENT

200 mg $K_3Fe(CN)_6$

50 mg KCN

1000 mg $NaHCO_3$

1250 mg in 1000 ml distilled water

The reagent should be stored, refrigerated in a sealed, dark glass bottle. In this manner it may be usable for 6 months or more.

APPENDIX IV

DETERMINATION OF HEMOGLOBIN CONCENTRATION

Figure 6. Sample hemoglobin concentration calculation. The line is constructed by plotting the % absorbance of each standard against its known hemoglobin concentration.

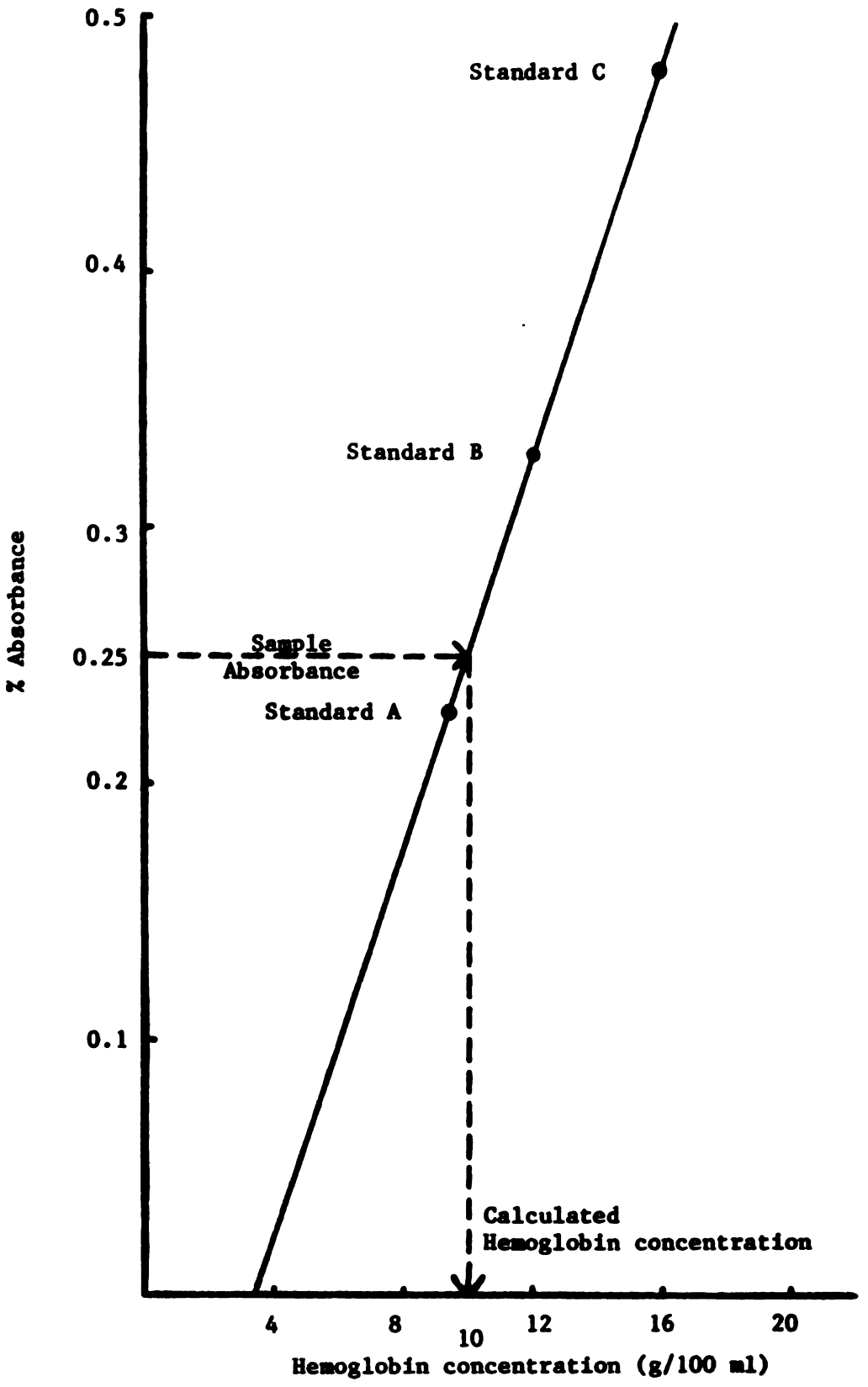

0.5
0.4
0.3
0.25
0.2
0.1
% Absorbance
Standard C
Standard B
Sample
Absorbance
Standard A
Calculated
Hemoglobin concentration
4
8
10
12
16
20
Hemoglobin concentration (g/100 ml)

Figure 6.

APPENDIX V

SCINTILLATION COUNTING

Reference: Instruction Manual, Beckman Liquid Scintillation Counter LS-100-C, Beckman Instruments, Fullerton, Ca.

Liquid scintillation counting provides for the determination of radioactivity in samples emitting radiation. The energy emitted by each sample is converted to light energy by the fluorescent compounds (fluors) in the scintillation fluid. The light energy is detected by a photomultiplier tube which is connected to amplifiers and a scaler circuit. The results are expressed as the number of counts of radioactive emissions detected per minute, that is counts per minute (CPM).

The counts detected are not always equal to the number of actual emissions of the isotope. This is due to chemical quenching, the absorption of some of the emissions by the chemicals present in the solution. It is important, therefore, to determine the efficiency of the scintillation system and calculate the actual degradations per minute (DPM) of each sample. The efficiency can be determined by detecting the CPM of samples with known DPM values, but with varying degrees of chemical quenching (Quenched C-14 Standard Set Beckman Instruments, Fullerton, California), and using the following equation:

$$\% \text{ Efficiency} = {}^{CPM}/_{DPM} \times 100 \qquad (87.24\% = \frac{173600}{199000} \times 100)$$

The Beckman LS100-C liquid scintillation counter offers built-in quench

calibration through an external standard determination. In addition to CPM, the counter printout records external standard values for each sample. For each set of unknowns to be tested a graph is constructed plotting the external standard value of each quenched standard against its calculated efficiency (Figure 7). The efficienty of the unknown samples is determined from their external standard values using this graph. The actual DPM of each unknown sample can be calculated using the following equation:

$$DPM = CPM/\%\ Efficiency \times 100 \qquad (1340.6 = \frac{1190.5}{88.8\%} \times 100)$$

The calculated DPM is more representative of the actual amount of radiation emission of each sample.

The major problem in counting whole blood samples is color quenching. The solubilized red cells exhibit a dark red color when in solution with the fluors due to the presence of hemoglobin. Part of the light energy produced by the fluors is absorbed by the solution due to its color, rather than being detected by the photomultiplier tube. This results in less efficient, lowered counts. If the colors of all samples are of unequal intensity, the efficiency of each sample is different. CPM's are, therefore, unrepresentative of the actual DPM occurring. This can partially be overcome by decolorizing each sample with a few drops of H_2O_2, but this only increases the chemical quenching with little change in color of whole blood samples. Quenching can also be corrected by preparing a standard set with constant radioactivity, but varying color intensities. The sample treatment is similar to that followed in correcting chemical quenching. A third method used to compensate for color quenching is to eliminate the source of the color

altogether. In this case, the latter method was chosen due to the relative ease of removing the hemoglobin from the red cells.

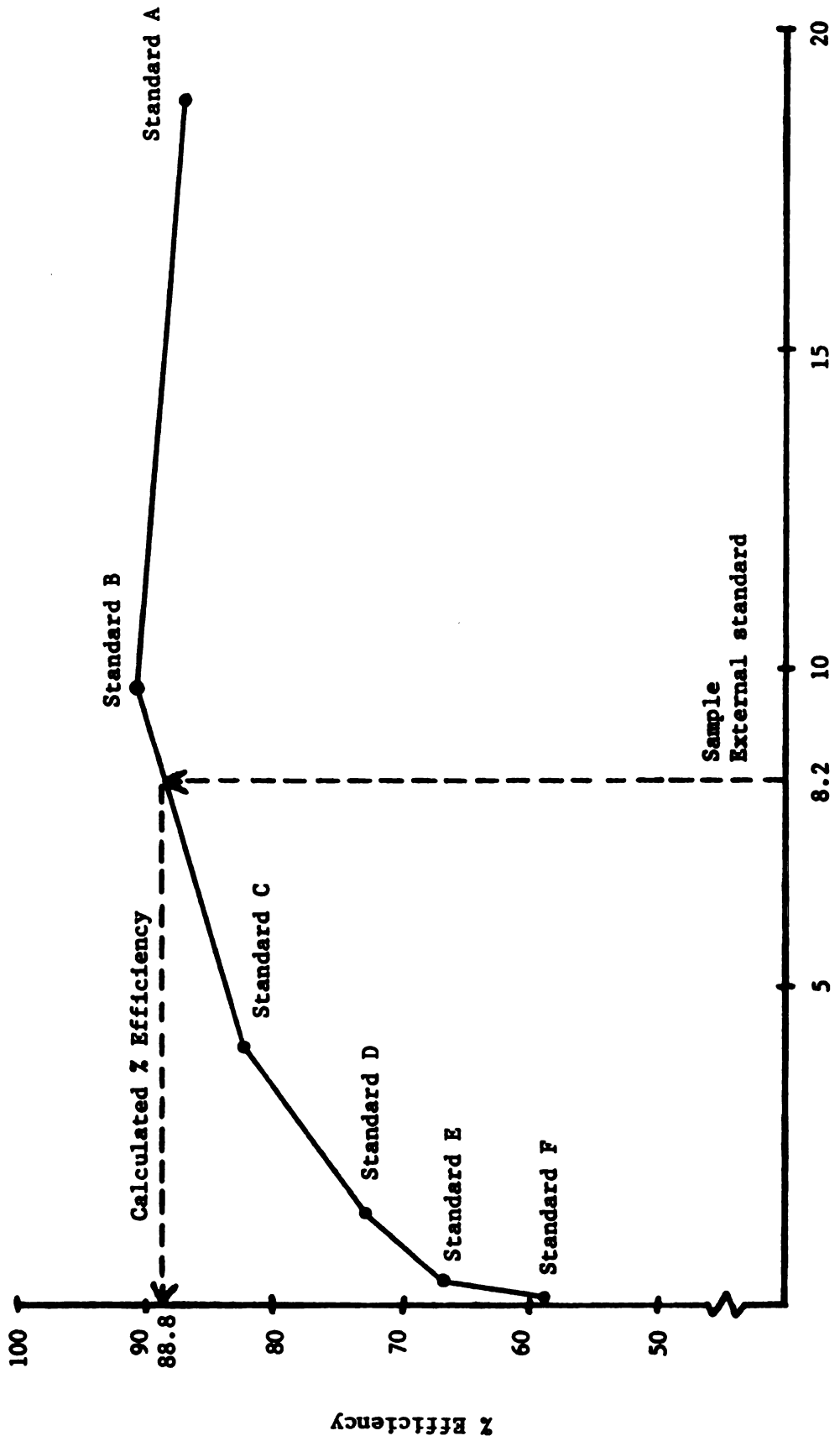
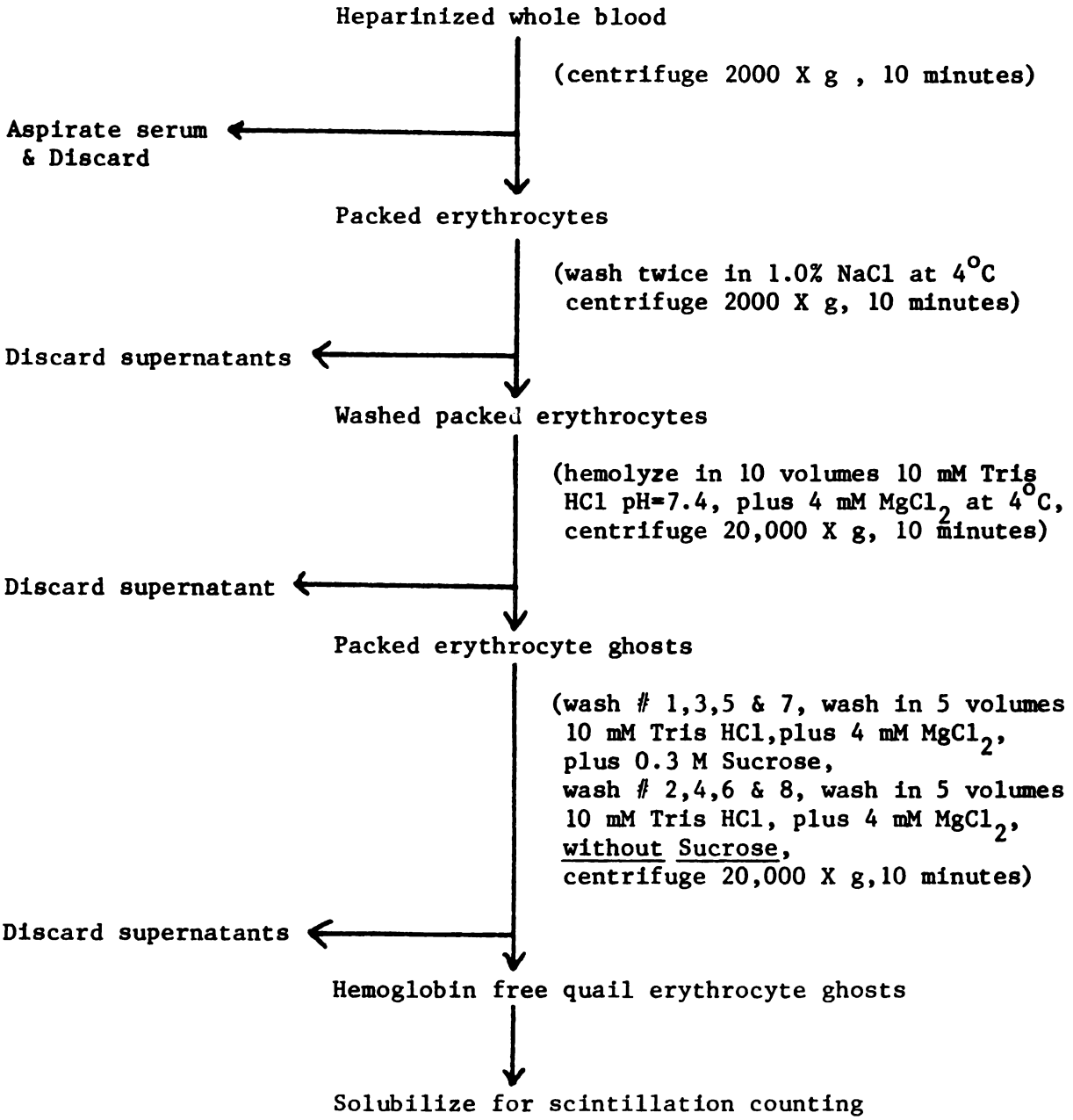


Figure 7. Sample % efficiency calculation

APPENDIX VI

ERYTHROCYTE GHOST PREPARATION

FLOW CHART



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