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IN DAIRY CATTLE

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James Harold Forsell

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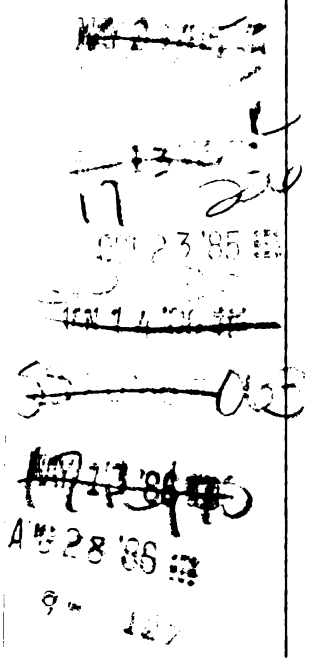

AN IMMUNOTOXICOLOGICAL EVALUATION OF PENTACHLOROPHENOL IN DAIRY CATTLE

By

James Harold Forsell

A DISSERTATION

Submitted to
Michigan State University
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ABSTRACT

AN IMMUNOTOXICOLOGICAL EVALUATION
OF PENTACHLOROPHENOL IN DAIRY CATTLE

By

JAMES HAROLD FORSELL

Pentachlorophenol (PCP) is an antimicrobial agent widely used for the preservation of wood. The immunotoxicity of a technical grade of PCP was evaluated in eight lactating Holstein-Friesian cattle. These animals were randomly allotted in pairs to either a control or a treatment group. The treatment group was fed technical PCP for 135 days (0.2 mg/kg body wt/day for 75 - 84 days followed by 2.0 mg/kg body wt/day for 56 - 62 days.

In a second study the immunotoxicologic and clinicopathologic effects of analytical and technical grade PCP were compared in dairy calves. Fifteen Holstein-Friesian bull calves were randomly assigned to one of five treatment groups: control, 1.0 mg and 10 mg analytical PCP/kg body wt/day, and 1.0 mg and 10.0 mg technical PCP/kg body wt/day, for 43 days. Assays conducted to evaluate lymphocytes included: 1) quantitation of serum immunoglobulins; 2) induction of blastogenesis *in vitro* using lymphocytes obtained from the blood and other lymphoid tissues; 3) enumeration of lymphocyte subpopulations; 4) quantitation of antibody formation induced by foreign antigens *in vivo*; 5) skin testing as a measure of delayed

hypersensitivity; and 6) histopathologic evaluation of lymphoid tissues. Neutrophil function was evaluated using latex particle phagocytosis and chemiluminescence. The clinicopathologic profile consisted of 43 tests in hematology, clinical chemistry and urinalysis. The results demonstrated no statistically significant differences between mature lactating control and penta-treated cattle; nor in calves fed either analytical or technical PCP at 1.0 mg/kg versus controls. Major findings in the calves exposed to 10 mg/kg technical PCP included marked thymic atrophy, decreased serum total protein, and elevation in serum gamma-glutamyl transferase (GGT). A decreased lymphocyte blastogenic response was seen in calves given 10 mg/kg analytical PCP.

A third aspect of this research dealt with establishing the conditions required by bovine neutrophils to change shape from a round to a bipolar form. PCP eliminated the shape change response *in vitro* at levels of 0.1 mg/ml.

Lastly, a technique was established and validated for isolating viable hepatocytes from cattle. This procedure was designed as a xenobiotic metabolism and activation system for use in co-culture with bovine lymphoid cells for *in vitro* immunotoxicity testing.

To Harold and Irene,
my parents

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Perhaps as important as anyone in a graduate program, is one's fellow graduate students. In this regard, I acknowledge Donald Kirsch, Brian J. Hughes and John H. Kinzell.

I also acknowledge the assistance of Edward W. Sparrow Hospital's Immunology Laboratory in providing technical skills for many of the immunologic assays used in these studies.

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INTRODUCTION

Pentachlorophenol is a broad-spectrum biocide, due to its ability to uncouple oxidative phosphorylation in many species, including man (EPA, 1980). Additional toxicity may result from the dioxins and other contaminating chemicals present in pentachlorophenol (EPA, 1980).

Most of the greater than 40 million lbs/yr of pentachlorophenol produced in the Unites States is used as a wood preservative (EPA, 1980). Pentachlorophenol-treated wood is often used in construction of livestock housing and feeding facilities. Livestock housed in such facilities may become contaminated with pentachlorophenol by ingestion, dermal contact and inhalation.

In Michigan, reports of poor health in dairy cattle have been attributed to exposure to pentachlorophenol (Thomas et al., 1977). Among the clinical signs reported, are decreased milk production, poor general appearance, skin lesions, increased incidence of mastitis, persistent infections, high calf mortality and death (Thomas et al., 1977). Many of these signs suggest a suppression of immune function.

Prior to this research, the question of immunomodulation as a consequence of pentachlorophenol exposure in dairy cattle was not addressed. In fact, many of the methodologies needed to evaluate bovine immune function were undeveloped or unrefined prior to this study.

This dissertation describes methods which were developed and used for evaluation of immune function in cattle. These methods were then used to evaluate immune function in lactating dairy cattle and calves orally exposed to pentachlorophenol.

A xenobiotic metabolism system was also developed for use with chemicals needing activation before proper evaluation for toxicity. This system involved the isolation of bovine hepatocytes and their use in *in vitro* xenobiotic activation systems.

LITERATURE REVIEW

PENTACHLOROPHENOL

In 1930,chlorinated phenols were produced in order to test their efficacy as wood preserving agents. Pentachlorophenol, tested two years later, was found to be a superior wood preservative (EPA, 1980). The effectiveness of pentachlorophenol as a preservative resides in its ability to prevent wood biodeterioration caused by fungi, particularly Basidiomycetes, which have the ability to enzymatically degrade the structural cellulose and lignin in wood. In addition to its use as a wood preservative, pentachlorophenol is useful in control of wood-destroying insects, as a herbicide defoliant, a mossicide and a mushroom house biocide.

Pentachlorophenol is a toxicant in bacteria, fungi and insects because it uncouples oxidative phosphorylation in living cells. Since oxidative phosphorylation is similar for the aerobic generation of adenosine triphosphate in all biologic systems, pentachlorophenol is a highly operative broad-spectrum biocide.

In 1974, approximately 54 million pounds of pentachlorophenol were produced in the United States (Fuller et al., 1977). In 1978 about 43.6 million pounds of pentachlorophenol were used in the treatment of wood products. This level of production has been maintained.

Commercial grade pentachlorophenol is 80% pentachlorophenol, 6% tetrachlorophenol isomers, and 6% other chlorinated phenols. The remainder of the pentachlorophenol is composed of other chlorinated compounds including dibenzo-p-dioxins and dibenzofurans. Before it is used, pentachlorophenol is dissolved in a petroleum solvent. A water soluble pentachlorophenol is available as the sodium or potassium salt.

The presence of the dibenzo-p-dioxins and dibenzofurans in pentachlorophenol has generated considerable interest due to the known toxicity of these chemicals and the concern for their contribution to overall pentachlorophenol toxicity (Federal Register, 1978). It was because of the presence of dioxins in pentachlorophenol and the extensive use of this pesticide which caused it to be placed on notice of "Rebuttable Presumption Against Registration" (RPAR) by the Office of Pesticide Programs of the Environmental Protection Agency (Federal Register, 1978). Such a notice was issued on October 18, 1978, when the EPA determined there was evidence of sufficient risk in the use of a given pesticide to warrant a review of the advisability of its continued use.

The RPAR review process starts only after the existence of a risk (trigger) has been demonstrated by the Environmental Protection Agency. In this case, the pentachlorophenol trigger was EPA's determination that pentachlorophenol may exceed risk criteria relating to teratogenic and fetotoxic effects in mammalian tests species (Federal Register, 1978). The information used as a basis for the trigger is compiled and published in the Federal Register which is the formal notice of RPAR. This document is referred to as a Position

Document One or PD1. The public is then given a period of time whereby interested parties may rebut the risk data addressed in the PD1.

The outcome of the rebuttal determines whether the chemical is returned to registration, a move which results in the publishing of a Position Document Two (PD2) in the Federal Register; or a risk-benefit analysis and a proposed regulatory position are published in what is referred to as a Position Document 2/3 (PD2/3). The latter situation results when the rebuttal is not successful. A PD2/3 document has been published for pentachlorophenol (Federal Register,1981). The PD2/3 document is then reviewed by various agencies leading to the publication of a Position Document Four (PD4) which contains the final regulatory action to be taken on a chemical. The PD4 on pentachlorophenol has not as yet been published.

Pentachlorophenol is synthesized through the direct chlorination of phenol using catalysts such as $FeCl_3$, $AlCl_3$, and $SbCl_3$ with heat. The process is a two-step reaction in which the first stage involves raising the reaction temperature to 105^{o}C to form tri- and tetrachlorinated phenols. The subsequent step involves increasing the reaction temperature to keep the mixture molten in order to chlorinate the lower chlorinated phenols to the fully chlorinated phenols (Cirelli, 1978). It is during the second, high temperature stage that various dioxin and furan congeners found in pentachlorophenol are formed. These impurities are responsible for the buff-colored crystals characteristic of the commercial

or technical grade of pentachlorophenol (tPCP).

Pentachlorophenol preparations are available with reduced levels of dioxins and furans. Dow Chemical Co. developed a procedure to produce pentachlorophenol with significantly reduced levels of impurities. Dow calls their improved pentachlorophenol Dowicide EC-7^{R}. Analytical grade pentachlorophenol (aPCP) is also available. Table 1 compares the composition of the different grades of pentachlorophenol.

The final physical form of pentachlorophenol after manufacture includes pellets, prills, and one-half ton blocks. Pentachlorophenol treating plants receive pentachlorophenol bags, bulk and solid blocks and cut the pentachlorophenol with petroleum solvents to a 5-7% solution. Solvents used to dissolve pentachlorophenol vary; typically they include kerosene, mineral spirits, number 2 fuel oil, methylene chloride or liquefied petroleum gas (Cirelli, 1978).

The wood preservation process is either a pressure or non-pressure process with 95% of the wood being treated with the pressure system. The pressure treatment of wood results in retentions of 20 to 30 pounds of preservative per cubic foot for the full-cell process and 6-12 pounds per cubic foot for the empty cell process (EPA, 1980). Non-pressure wood preservation methods include thermal, brush, dip, spray, diffusion, vacuum and cold soak methods.

The estimated production of pentachlorophenol-treated wood in 1978 was 80 million cu. ft. Most of the pentachlorophenol-treated lumber (93%) was classified as poles, fence posts,

Table 1

Component Analysis of Three Grades of Pentachlorophenol

Chlorophenols (%)	Commercial[a]	Improved[b]	Analytical[b]
Pentachlorophenol	85-90	88-93	99.02
Tetrachlorophenol	4-8	7-12	.98
Trichlorophenol	0.1	0.1	--
Other	2-6	0.1	--
Chlorodibenzo-p-dioxins (ppm)			
Octa	1000[c]	2-30	1.2
Hepta	378	2-3	1.8
Hexa	173	0.2-1.0	0.2
Tetra	0.035[d]	--	--
Chlorodibenzofurans (ppm)			
Octa	54-182[a]		
Hepta	37-116		
Hexa	6.8-7.1		
Penta	0.9-1.1		
Tetra	0.2-0.7		
Tri	0.02-0.05		
Hexachlorobenzene	30-80 ppm		
Polychlorinated Biphenyls	1-2 ppm		
Chlorinated diphenyl ethers[e]	100-1000 ppm		

[a]Analysis reported by Monsanto Chemical Company, St. Louis, MO, personal communication, Dr. Dan Roman, 1978.

[b]Analysis of Dowicide EC-7(R) as reported by Dow Chemical Co., Midland, MI: personal communication, Dr. Robert L. Johnson, 1980.

[c]Analysis as reported by Kinzell et al., 1981.

[d]Does not include 2, 3, 7, 8-TCDD.

[e]Nilsson et al., 1978.

lumber and timbers. Little pentachlorophenol-treated wood (0.5%) is used for crossties, switch ties, and landscape ties, products which are traditionally treated with creosote solutions (EPA, 1980).

The use of pentachlorophenol (penta) treated lumber in farms in Michigan has been surveyed by Shull et al.,(1981). Penta-treated wood was found in 47.2% of the main livestock buildings on the farms surveyed. In 21.3% of these buildings the extent of penta-treated wood use was classified as medium to extensive. The most prevalent use of penta-treated wood was for pole supports (45%) and splash boards (40.5%). However, 4.8% of calf pens, 15.8% of feed bunks, 9.3% of corn cribs, 8.4% of bunker silos, 4.1% of hay racks. 0.9% of mineral feeders and 0.7% of feed bins were constructed of treated wood. Since treated wood is recommended for use only where wood is exposed to excessive moisture or soil, excluding feed contact surfaces, these authors concluded that treated wood is being used excessively and improperly on many Michigan dairy farms.

Chemical and Physical Properties

Pentachlorophenol (C_6HCl_5O) is a fully chlorinated phenol (Figure 1). In its pure form, PCP is a white solid having a needlelike crystalline structure. PCP has a very pungent odor, especially when hot. It is almost insoluble in polar solvents but freely soluble in alcohol and ether. The solubility of PCP in various solvents is shown in Table 2.

PCP is a weak acid with pKa of 4.74 (EPA, 1980). It exists as a nonpolar molecule under acidic conditions and as a polar anion under basic conditions. Thus, PCP reacts with

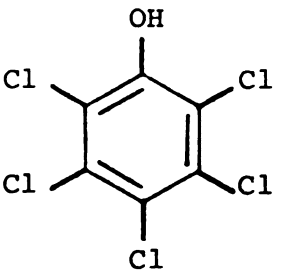


Figure 1. Pentachlorophenol

strong alkali bases such as sodium hydroxide to form the water-soluble salt which is soluble at greater than 0.4g/100g water at pH 8.0 (Myeling and Pitchford, 1966). Pentachlorophenol is quite stable at elevated environmental temperatures and is rather chemically inert. It is not subject to the easy oxidative coupling or electrophilic substitution reactions common to most phenols. However, photodegradation does occur, apparently, by free-radical pathways (Plimmer, 1970).

Volatility of PCP is relatively low, but losses do occur from soil, water and treated wood. The physical-chemical properties of PCP are given in Table 3.

Exposure to Pentachlorophenol

Pentachlorophenol can be absorbed during oral, respiratory and dermal contact. The data supporting the actual kinetics involved with the different routes of PCP exposure are scarce, and subject to disagreement. Many factors are used in order to estimate exposure levels. Thompson et al.,(1979) found that the maximum expected concentration of PCP in air in a barn constructed in part from penta- treated wood was 0.02 mg/m^3. Using estimated values for a 24 hour total air volume which could

Table 2

Solubility of Pentachlorophenol

Solvent	g/100g solvent (20-30°C)
Water	0.0014-0.0019
Carbon Tetrachloride	2-3
Benzene	11-14
Xylene	14-17
Acetone	21-33
Ethanol	47-52
Diethyl Ether	53-60
Methanol	57-65

Bevenue and Beckman, 1967.

Table 3

Physical-Chemical Properties of Pentachlorophenol

Color	White
Molecular weight	266.26
Melting point	190-191°C
Boiling point decomposes	309-310°C
Density at 22°C referred to water at 4°C	1.978 g/cm^3
Vapor pressure 20°C	0.00011 mmHg
100°C	0.12 mmHg

Modified from Bevenue and Beckman, 1967.

be inhaled by a 514 kg cow and assuming a 100% retention of all PCP entering the lungs; a total exposure of 3.138 mg/cow or 0.006 mg/kg can be calculated (EPA, 1980). This level of exposure would result in an estimated blood level of 0.013 mg/liter (ppm), (Osweiler et al., 1977).

PCP exposure by the oral route would occur as a result of eating contaminated feed or by licking and chewing PCP-treated lumber. Penta-treated wood contains 10 to 16 grams of penta per board foot (bd. ft = 12 X 12 X 1 in) (EPA, 1980). A cow weighing 514 kg would have to consume an average of 0.039 bd.ft/day to receive a penta exposure of 1 mg/kg, 0.078 bd.ft/day for 2 mg/kg, 0.39 bd.ft/day for 10 mg/kg, and 0.78 bd.ft/day for 20 mg/kg. If 0.39 bd.ft was eaten/day, a daily exposure of 10 mg/kg would require the eating of 56 cubic inches of wood each day.

The exposure which may result from licking the oozing (bleeding) material from freshly treated lumber or by eating feed into which this material has migrated can similarily be estimated. Wood preserving solutions usually contain 5 to 7% pentachlorophenol (EPA, 1980). At 6% PCP , it would require ingestion of 9 ml of the bleeding material to obtain a 1 mg/kg exposure level.

Feeding adult lactating cattle 0.2 mg/kg or 2.0 mg/kg/day tPCP resulted in steady state blood levels of 2.9 and 12.5 ppm, respectively (Kinzell, 1981). Nonlactating heifers fed various grades of pentachlorophenol at 15 mg/kg had serum PCP concentrations of 32.8 to 86.9 ppm (Parker et al., 1980). Calves given 1.0 mg/kg or 10 mg/kg PCP, incorporated

in milk, had blood levels of 32 to 92 ppm (Hughes, 1982). The nonlinear relationship between the amounts of PCP ingested and the level of PCP in the blood indicates that absorption from oral exposure is not 100%, and varies considerably.

PCP in the blood of dairy cattle housed in total confinement-free stall pole barns in which penta-treated wood was used extensively reached 0.004 to 0.033 mg/kg. In one barn where penta-treated wood was used extensively, including the feed-bunk, everage blood PCP levels reached 0.3 mg/kg (Van Gelder, 1977). The data above provides the best available information concerning actual levels of PCP exposure in cattle in a farm environment.

Dermal absorption data are essentially non-existent for cattle and other food-producing animals. The implication that dermal exposure does occur in the farm environment comes from case histories of penta toxicosis in pigs where sows were farrowed on freshly treated lumber. Typically, the newborn pigs were affected and the sow was not. Volatilization of penta did not appear to be the problem, because covering the penta-treated floor resulted in the cessation of mortality (Schipper, 1961).

A case history reported by Ryan (1983) recorded the death of piglets within 24-48 hours of birth on a wooden floor which was freshly treated with penta. Analysis of the floor wood showed 3100 ppm PCP present. PCP residue analysis in the livers of two pigs resulted in no PCP detected at their maximum limit of detection (0.02 ppm). Although it is highly unlikely, dermal absorption of dioxins was implicated by the author as the cause of death in this instance.

Chickens are also affected by penta, showing increases in liver fibrosis and morbidity when exposed to penta (Ryan and Pilon, 1982). In these cases penta is usually present in sawdust or wood chips used for bedding (Curtis et al., 1974).

Determination of the level of animal exposure becomes a problem when a toxic chemical is common in the environment. Shull et al., (1981) revealed that penta-treated wood was present on nearly 50% of the dairy farms surveyed in Michigan. Penta was either used improperly or over-used in about 70% of these farms. It is clear that the potential for animal exposure and toxicosis is present, and that penta residue problems exist.

Absorption of Pentachlorophenol

Absorption of toxicants can take place along the entire length of the gastrointestinal tract from the mouth to the rectum. Most toxicants cross body membranes by simple diffusion. The rate of transfer is dependent on lipid solubility and concentration gradient across the membrane (Klaassen, 1980).

If a toxicant is a weak organic acid or base, the degree of its ionization in various portions of the gastrointestinal tract is important in determining the extent of absorption. The degree of ionization of a chemical is dependent upon the pKa of the chemical and the pH of the medium of each particular region of the gastrointestinal tract. PKa and pH are important because only the non-ionized (lipid soluble) form can be transported across biologic membranes by diffusion (Klaassen, 1980).

Pentachlorophenol is classified as a weak acid with a pKa of 4.74 (EPA, 1980). Using the Henderson-Hasselbach equation for an acidic chemical (pH=pKa + log ionized / non-ionized),

the ratio of the non-ionized form of PCP to the ionized form can be calculated. The ratio thus calculated is 1 to 1000 in favor of the lipid-soluble (non-ionized) form.

In an intestinal environment with a pH of 6, the ionized to non-ionized ratio would become 10 to 1 respectively. Under the latter conditions much less PCP will be absorbed, because only 10 percent of the PCP is in the absorbable, non-ionized form at any time. Thus, PCP is in the non-ionized lipid-soluble form in the stomach and subsequently tends to be absorbed very well. However, because of the very large surface area of the intestine, the overall capacity of the intestine for chemical absorption is greater than would be expected based on common ion effects alone.

The skin provides a relatively good barrier against many toxic agents. For a chemical to be absorbed by the percutaneous route,it must pass through the outer densely packed layer of horny, keratinized epidermal cells (stratum corneum). This layer of the skin forms the rate-limiting barrier for the cutaneous absorption of toxicants. Toxicants are absorbed by passive diffusion (Klaassen, 1980).

Incidents where dermal absorption of PCP has caused death in humans have been recorded. Two cases of fatal poisoning occurred in a hospital nursery where pentachlorophenol had been used as a fungicide in the laundry room. The infants contacted the PCP through their diapers (Armstrong et al., 1969).

In contrast to the skin, in which a chemical must pass through a large number of cells, toxicants absorbed by the lung may pass through only two cells. Toxicants that are absorbed by

the lung are usually gases, volatilized liquids and aerosols. Particles with sizes in excess of 5 um do not pass beyond the nasopharyngeal region. The vapor pressure of PCP is 0.00011 to 0.12 mmHg at 20° and 100°C, respectively (Benvenue and Beckman, 1967). At 20°C the vapor pressure corresponds to a theoretical air vapor density of 1.5 mg/m^3. In a 514 kg cow, this exposure level translates to a maximal theoretical PCP exposure via the respiratory route of 0.46 mg/kg/day. As environmental temperature rises toward 100°C, the resulting increase in vapor pressure could substantially increase exposure.

Radiotracer studies using carbon-14-labeled pentachlorophenol (^{14}C-PCP), demonstrated that absorption of PCP is rapid and extensive regardless of the route of exposure. Braun et al., (1977) gave single oral PCP doses of 10 and 1000 mg/kg to rats. Plasma levels of ^{14}C increased rapidly, peaking within the first six hours. In Rhesus monkeys given a single oral dose of ^{14}C-PCP, the ^{14}C-PCP took 12 to 24 hours to reach maximum concentrations (Braun and Sauerhoff, 1976). Harrison (1954) reported that when sheep were force-fed PCP-impregnated sawdust, they reached maximum levels of PCP in the blood within 3 to 6 hours. Kinzell (1981) showed that it takes about 10 hours for a single 0.2 mg/kg dose of ^{14}C-PCP to reach a maximum serum concentration in a cow. Kinzell gave the cow 0.2 mg/kg/day of cold PCP for 95 days before administering the ^{14}C-PCP dose; the cold-dosing was then resumed. The cattle given 0.2 mg/kg PCP/day required three days to reach steady state blood levels (Kinzell, 1981). Essentially, all PCP studies indicate rapid absorption of PCP into the bloodstream

after exposure from any route.

Distribution of Pentachlorophenol

When PCP reaches the bloodstream it is bound to serum proteins, primarily albumin (Hoben et al., 1976). The PCP is then circulated to the various body compartments. Although some species variation exists, most studies show similar results in the distribution of PCP in tissues. Disregarding serum and urine PCP values which fluctuate enormously depending on the length of time after exposure, the tissues which consistantly rank one and two for the highest residue levels of PCP are liver and kidney. Lower concentration of PCP occur in the gall bladder, lung, stomach and intestine. The lowest PCP levels are found in the fat, muscle and brain (Walters, 1952; Jakobsen and Yllner, 1971; Larsen et al., 1972; Braun and Sauerhoff, 1976; Braun et al., 1977; Kinzell, 1981; Hughes, 1982).

Since PCP is found in the gall bladder, liver and intestines, enterohepatic circulation of PCP is indicated. High levels in the stomach indicate that either gastric secretion of PCP is occurring, or the stomach is a major site of PCP absorption. Placental transfer of PCP has been shown in cattle (Hughes, 1982). Hughes found that the serum level of PCP in the calf was 32 to 38% of the serum PCP level in the dam. Hinkle (1973) reported placental transfer of PCP in the hamster, and placental transfer of PCP has been noted in the rat (Larsen, 1975).

Metabolism of Pentachlorophenol

Pentachlorophenol metabolism studies in experimental animals have shown the following metabolites: PCP glucuronide

conjugate, tetrachlorohydroquinone (TCH) and the glucuronide conjugate of tetrachlorhydroquinone (Figure 2). A congener of PCP (2, 3, 4, 6 tetrachlorophenol) is present in penta at up to 12% by weight, and has been shown to be excreted unchanged in rats (Ahlborg and Larsson, 1978).

In most species studied, PCP is excreted as the parent compound. No metabolism was evident in monkeys given a single oral dose of ^{14}C-PCP (Braun and Sauerhoff, 1976). In rats, studies have shown PCP metabolites to break down this way; 43% unmetabolized, 49% PCP conjugate, 3% TCH and 3% the TCH conjugate (Ahlborg et al.,1974) and in a study by Braun et al.,(1977), 75% unmetabolized, 10% PCP conjugate and 15% TCH. In the mouse, Ahlborg et al.,(1974) found 41% of the PCP to be unmetabolized, 13% converted to PCP conjugate, 24% to TCH and 22% to the TCH conjugate.

Very little is known about the metabolism of PCP in humans and in large animals. It appears that the metabolism of PCP is inducible. The conversion of PCP to TCH is induced after treatment with phenobarbital (Ahlborg et al., 1978). Also, the formation of the metabolite TCH is inhibited when SKF 525-A, a known inhibitor of several microsomal enzymes, is used.

Excretion of Pentachlorophenol

Urine is the main route of excretion for pentachlorophenol. Feces and milk are minor routes of excretion. Only trace amounts of PCP, less than 0.05% of administered dose, was reported as expired in the air (Larsen et al., 1972; Jakobson and Yllner, 1971).

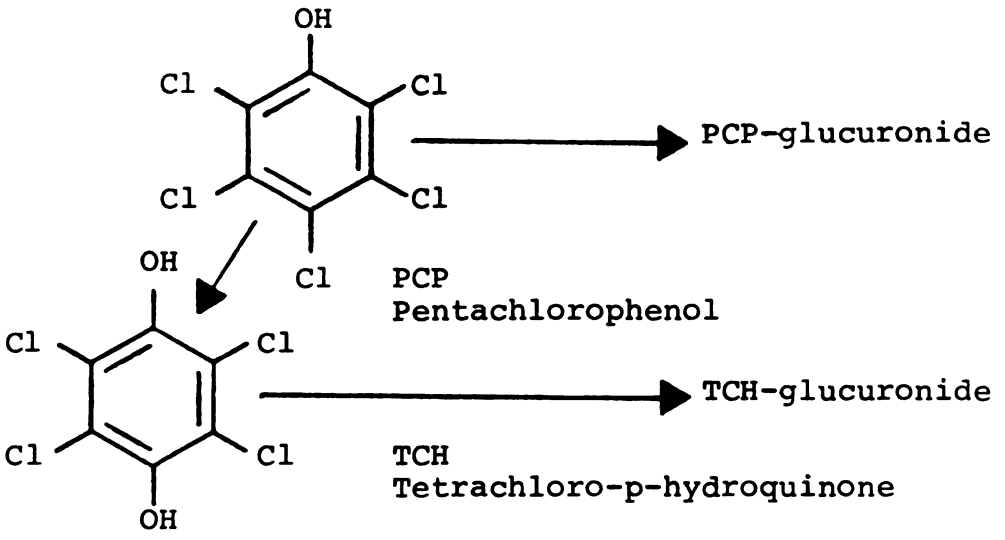


Figure 2. Metabolism of Pentachlorophenol

Typically, mice have been shown to excrete 68 to 83% of PCP administered either orally, subcutaneously, or intraperitoneally in the urine in 4 - 10 days. Another 8 to 21% was excreted in the feces (Larsen et al., 1972; Jakobson and Yllner, 1971). In rats, excretion of PCP in the urine ranges from 50% to 80%. Excretion of PCP in feces ranges from 19% to 43% (Braun et al., 1977; Ahlborg et al., 1974). The ranges observed reflect differences in sex, route of administration, length of collection and dose of PCP. Rhesus monkeys given 10 mg/kg ^{14}C-PCP (Braun and Sauerhoff, 1976) excreted 70 to 75% of the dose in urine and 12-18% in feces. The plasma half-life of penta in humans is given by Braun et al.,(1978) as 30 hours. Braun further stated that 86% of an oral dose of PCP is eliminated in urine. In the cow, 75% of a ^{14}C-PCP dose (0.2 mg/kg body wt/day) was excreted in the urine in 76 hours (Kinzell, 1981). An additional 5% was detected in the feces. Both

absorption and elimination followed first-order kinetics with half-lives of 4.28 and 42.8 hours, respectively.

Excretion of PCP in the milk of cows has been reported by Firestone (1979), Kinzell (1981), and Hughes (1982). Kinzell determined that 5% of PCP was eliminated in the milk of the mature cow. Hughes (1982) found that PCP concentrations in milk were 7 to 10% of those found in bovine serum.

It appears that PCP is primarily excreted in the urine. No significant secretion or reabsorption of PCP is believed to occur in the kidney (Braun and Sauerhoff, 1976).

Enterohepatic circulation of PCP is indicated as previously mentioned. Enterohepatic circulation of PCP would account for the constant low levels of PCP found in the feces of the different species studied; as unabsorbed PCP would be eliminated as a bolus in a shorter time period.

Toxicodynamics of Pentachlorophenol

As is typical of all uncouplers of oxidative phosphorylation, the clinical signs of acute PCP intoxication are nausea, gastric upset, restlessness, sweating, rapid respiration, tachycardia, fever, cyanosis, thirst, loss of weight, and finally collapse and death associated with a rapid onset of rigor mortis (Murphy, 1980). Irritation of the skin, eyes, nose, throat, and respiratory tract may also occur. The severity of these symptons is increased by environmental and physiological factors such as higher ambient temperature, decreased renal function and poor health. No specific treatment is available. Supportive therapy given concurrently with lowering of the body temperature

can be helpful. Prognosis is good if death does not occur in the first 24-48 hours.

The oral LD_{50} of PCP is 146-175 mg/kg in rats, 120 mg/kg in sheep, and 140 mg/kg in calves (Gaines, 1969). This would place PCP into the general category of a moderately toxic substance, based on its acute LD_{50} (Buck et al., 1976).

Chronic exposure to PCP has resulted in feed refusal, increased neonatal mortality and decreased body weights in pigs confined in penta-treated farrowing pens (Schipper, 1961), and decreased body weight and feed efficiency in heifers (McConnell et al., 1980). Pathologic findings in the above studies were,lesions on the mucosal surface of the stomach, mild emphysema, congestion in lungs, enlargement of lymph nodes, inflammation of small intestines, infarcted areas in the liver and spleen and hemorrhage in the kidneys of pigs. In the heifers there was an decrease in thymus weight, progressive anemia, villous hyperplasia of the urinary mucosa, hyperplasia of the gall bladder as well as the bile ducts, and keratin deposition in the Meibomian gland of the eyelid. The level of exposure in the heifers (McConnell et al., 1980) was 15 mg PCP/kg/day. The treatment groups received various amounts of tPCP as a portion of the 15 mg/kg/day total. McConnell concluded that the lesions observed were due to and characteristic of dioxin toxicosis.

In other studies which used lower amounts of PCP, few adverse effects were noted. Kinzell et al., (1982) observed no adverse effects on feed intake, body weight, and milk production in cows administered 2 mg/kg tPCP daily. However,

Kinzell did note enlarged liver, lungs, kidney and adrenals upon post-mortem examination. Herdt et al., (1951) observed no effects on calf behavior or in hematological parameters in calves given 7.6 mg/kg sodium pentachlorophenate per day in water. Likewise, the post-mortem examination was normal.

Deaths have occurred in humans as a result of acute PCP exposure. An incident in a hospital in St. Louis, Mo. in 1967 resulted in two infant deaths and several others temporarily affected when the hospital laundry used pentachlorophenol as a fungicide. The infants were contaminated by dermal exposure to nursery diapers and other linens (Armstrong et al., 1969; Barthel et al., 1969; and Robson et al., 1969). Residues in the linens ranged from 11.5 to 1,950 ppm. PCP in the tissue of one of the dead infants was 20-34 ppm in kidney, adrenal, heart, fat and connective tissue. One ill infant had a serum PCP level of 118 ppm. This was subsequently reduced to 31 ppm after an exchange transfusion. Serum PCP levels in nurses working during the time of the accident were 1 to 12 ppm.

Chronic PCP exposure in humans also occurs. Survey data accumulated by Cranmer and Freal (1970) revealed that the general population had urinary PCP levels of 6 to 11 ppb. Occupational exposure to PCP by employees engaged in the treatment of wood has been monitored (Klemmer et al., 1980). Mean serum PCP levels observed in the group with the highest exposure were 3.78 ppm. The only adverse effects observed were low-grade infections or inflammations of the skin and subcutaneous tissue,

of the protective membrane of the eye, and of the upper respiratory tract's mucous membrane. However, no cause-and-effect could be established between these effects and PCP exposure. Also, there seemed to be no long-term effects in the PCP exposed group. During the renal function testing the group of 22 workers had a mean of 2.3 ppm PCP in their urine for the year period. Renal function tests, including creatinine clearance and phosphorus reabsorption, were depressed while the workers were on the job, but showed significant improvement following a non-exposure period (Begley et al., 1977). In cattle, Kinzell (1981) found chronic interstitial nephritis and subacute urocystitis as the major pathologic changes in penta-treated cattle. In vitro testing of the kidney slices confirmed significant loss of renal function. However, Kinzell indicated that the relationship of lesions to the administration of penta was not clear. He stated that the effect could be related to the use of urethral catheters, causing an inadverdent introduction of infectious microorganisms into the bladder.

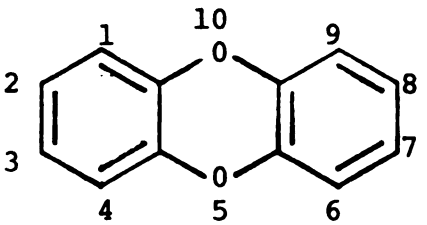
Hepatic porphyria has also been attributed to technical pentachlorophenol exposure (Goldstein et al., 1977). Goldstein fed tPCP at 20 ppm to rats and produced porphyria along with increased hepatic aryl hydrocarbon hydroxylase (AHH) and cytochrome P-450 activity, and increased liver weight. Because analytical PCP did not produce these effects, they were attributed to the contaminants in the penta used. In contrast, McConnell et al., (1980) did not observe hepatic porphyrin

changes or changes in urinary excretion of uro- or coproporphyrin in cattle fed up to 15 mg/kg day tPCP. McConnell et al. (1980) did, however, observe increased AHH activity as well as cytochrome P-450 levels. These changes were also attributed to the contaminants in penta.

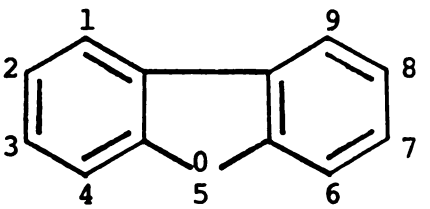
Contaminants in Penta

The chlorinated dibenzo-p-dioxins (CDDs) and chlorinated dibenzofurans (CDFs) in penta appear to be responsible for most of its toxicity. The major congeners of CDD found in penta include hexachlorodibenzo-p-dioxins (HxCDD), heptachlorodibenzo-p-dioxins (HpCDD), and octachlorodibenzo-p-dioxin (OCDD). Tetrachlorodibenzo-p-dioxin (TCDD) does occur in penta (Kinzell, 1981), however the specific congener 2, 3, 7, 8-TCDD does not occur. For the numbering system used for dibenzo-p-dioxins and dibenzofurans see Figure 3.

The degree of toxicity associated with dioxins varies with the amount and position of chlorination on the molecule. Typically, the more a dioxin is chlorinated, the more "inert" and less toxic it becomes. The LD_{50} of OCDD has not been determined, but it is very high. Johnson (1973) gave female rats up to 4 g/kg OCDD and failed to kill any of the rats. In comparison, HxCDD has two less chlorines and has a LD_{50} in rats of around 100 mg/kg (Johnson, 1973) and TCDD has been reported to have LD_{50}s in rats in the range of 0.022 and 0.045 mg/kg. The absorption of the CDDs also varies with the degree of chlorination. Norback el al. (1973) fed rats OCDD at 100 mg/kg and found 95% of the chemical in the feces and 4% in the urine.



Dibenzo-p-dioxin



Dibenzofuran

Figure 3. Numbering System for Dibenzo-p-dioxins and Dibenzofurans.

Tissue levels of OCDD were highest in fat, liver and skin. In cattle, OCDD is not absorbed very well, (Firestone et al., 1979) the OCDD which is absorbed accumulates in the fat and liver (Firestone et al., 1979; Parker et al., 1980).

Dioxin exposure causes chloracne in the skin of man, non-human primates, and rabbits (Nicholson and Moore, 1979). Chloracne is a rather severe type of dermatitis and appears as an acne-like eruption. Dioxins have also been implicated as carcinogens. TCDD has been reported to be carcinogenic when fed to rats at 0.1 ug/kg/day for two years (Kociba et al., 1978). However, other studies with TCDD have not shown carcinogenic activity (Innes et al., 1969); Berry et al., 1978).

The potency of TCDD as a carcinogen in rats is interesting in light of studies by Poland and Glover (1979) which failed to demonstrate any covalent binding of TCDD. Also, there is inconclusive evidence that TCDD is even a weak mutagen (Wasson et al., 1977). Since most chemical carcinogens are mutagenic and bind covalently to macromolecules, Pitot and coworkers (1980) suggested that the liver cancer associated with

chronic administration of TCDD might arise from the promoting activity of TCDD. Pitot et al., (1980) subsequently showed that TCDD is a potent tumor promotor in a multistage hepatocarcinogenesis system in the rat.

Chlorinated dibenzo-p-dioxins and dibenzofurans are potent inducers of the cytochrome P-450-mediated microsomal monooxygenases, and in particular aryl hydrocarbon hydroxylase (AHH) (Nicholson and Moore, 1979). AHH activity is mediated by the reversible stereospecific binding of TCDD to a cytosol receptor protein. The presence of the receptor is determined by the Ah locus in mice. It appears that TCDD binding to the receptor is responsible for the induction of AHH activity, as well as mediating TCDD's toxic responses in mice; (i.e., thymic involution and cleft palate formation) (Poland et al., 1979; and Poland and Glover, 1980).

Residual CDDs in animals and animal products is a problem. Firestone et al. (1979) reported the occurrence of dioxins in cow's milk. The levels of dioxin in the milk and body fat were 1000 times those in blood during exposure. The average daily excretion of HxCDD, HpCDD and OCDD in the milk was 33%, 3% and 0.6%, respectively, of the daily intake of dioxins as part of a penta dose. The low level of OCDD excretion in the milk is largely due to its low absorption from the rumen.

One hundred days after penta feeding was stopped (Firestone et al., 1979), appreciable levels of dioxin were still detected in the fat of cows. The levels of dioxin detected were comparable to that found in milk. The authors estimated that the half-life of the different dioxin congeners in milk fat ranged

from 41 - 50 days. In this same experiment, Firestone et al., (1979) obtained evidence suggestive of some placental transfer of dioxins in cattle.

IMMUNOTOXICOLOGY

Introduction

At the present time, an acceptable protocol for immunotoxicity testing is not available. Guidelines for a rudimentary immunotoxicologic test profile which should be incorporated into a total toxicity evaluation scheme was outlined by the European Economic Community (EEC) in 1977.

The guidelines suggested by the EEC include protocols designed to evaluate the potential risk of test chemicals by determining the functional significance of any effects observed on lymphoid organs found in routine toxicological studies, and to obtain information on the target immune organ, cell type or function impaired. A paragraph taken from the EEC guidelines illustrates how the EEC suggests that the evaluation be performed.

> At the termination of a toxicity study, thymus, spleen, and lymph nodes should be weighed and examined microscopically. From these data and measurements of serum immunoglobulin fractions and counts of circulating lymphocytes, the conclusion should be reached whether the substance has an effect on the lymphoid system or whether or not specific function tests have to be performed.

Obviously, the intent of this statement is that immune function testing be done only if a problem is observed, and after the toxicity testing is completed. More specifically, further evaluation is suggested only after hematologic and

histopathologic studies have been completed. While it is true that all the procedures mentioned above are necessary, these tests may be incapable of detecting all but the most immunotoxic chemicals.

Generally, histopathologic changes as well as serum immunoglobulin levels alone are inadequate as indicators of immune function. This is especially true at lower levels of chemical exposure. The circulating leucocyte count and differential count then become the only remaining tests of immune function on which to base a decision of immune toxicant. While leucocyte number and morphology are fairly sensitive indicators of host well-being, they may be difficult to interpret, especially in light of normal serum immunoglobulins and lymphoid organ morphology.

Ultimately, this scheme can lead to one of three outcomes. First, the tests can give a false negative (immunotoxic) result for the chemical tested. Secondly, they can give a positive result, but provide little information as to mode of action. This outcome would necessitate setting up a whole new study based on minimal knowledge of the immune lesion. As a result the new experiment would be large and cumbersome and may only provide the investigator with the data which should have been obtained in a properly designed original experiment. The third outcome is relative. The chemical passes the tests and is characterized as a non-immunomodulating chemical. This outcome, however, depends on the profile used.

An alternative to this scheme is to have immune function

tests which are incorporated into and run along with the toxicologic testing. These tests should be designed to evaluate all major functions of the immune system, be easy to perform, and be relatively inexpensive and reproducible among labs.

One approach to immune function testing is being fostered by the National Institute of Environmental Health Science group at Research Triangle Park, NC (Dean et al.,1982). They propose a minimum panel of in vivo and in vitro assays to assess immunological competence and host resistance following chemical exposure. The assays described are designed to test cell-mediated immunity (CMI), humoral mediated immunity (HMI), and phagocytosis by macrophages in rodents (Table 4).

This assay panel is currently being evaluated by the National Toxicology Program in the chemical bioassay program. Another comprehensive panel of in vivo and in vitro assays is being evaluated by the National Institute of Environmental Health Sciences group. This panel of tests is to be used in detecting immunomodulation following chemical exposure and is outlined in Table 5. These procedures evaluate pathotoxicology, host resistance models, delayed-type hypersensitivity, CMI, HMI, macrophage function, and bone marrow progenitor cells in detecting immunotoxicity.

While a standardized approach to the immunotoxicologic evaluation of chemicals in animals seems to be a practical goal, there are some negative aspects. First, once a protocol is commonly accepted, it tends to inhibit introduction of new information and procedures into the system. Also, there is a

Table 4

Minimum Screening Panel for Defining Immunomodulation After Chemical Exposure in Rodents

Parameter	Procedures Performed
Clinical Pathology	Hematology profile - complete blood count (CBC) and differential
Pathotoxicology	Necropsy - weights of body, spleen, thymus, liver, kidney, brain
Host resistance	Susceptibility to transplantable syngeneic tumor at LD 10-20
Delayed (cutaneous type) hypersensitivity (DTH)	Skin testing with a T cell dependent antigen
Cell-mediated immunity (CMI)	Induction of blastogenesis with Concanavalin A (Con A), Phyto-hemagglutinin (PHA) or with allo-genic leucocytes in a one-way mixed lymphocyte culture (MLC)
Humoral immunity (HMI)	Immunoglobulin levels (IgG, IgM, IgA), antibody response to T cell dependent antigen and blastogenic response to lipopolysaccharide (LPS)

Modified from Dean et al., 1982.

Table 5

A Partial List of Procedures Available for Detecting Immunomodulation After Chemical Exposure

Parameter	Procedures Performed*
Clinical Pathology	Hematology profile - CBC and differential with morphology and platelets[+]. Chemical Pathology - CK, HBD, ALT, BUN, creatinine ACP, ALP, LD, CHS, albumin (A), globulin (G), A/G, total protein and complement activity[+].
Pathotoxicology	Necropsy - weights of body, spleen, thymus, liver,kidney, brain[+] Histology - liver, thymus, adrenal, lung, kidney, heart, spleen, lymph node[+] and bursa[+] (avian species).
Host resistance	Tumor assays - tumor cell challenge TD 10-20, Endotoxin hypersensitivity LD 10-20, Resistance to parasites.
Delayed hypersensitivity	Skin testing with a T cell dependent antigen, T cell enumeration[+].
Lymphocyte proliferation (clonal expansion)	One-way mixed leucocyte culture, Mitogens - PHA, Con A, LPS.
Humoral immunity	Immunoglobulin levels (IgG, IgM, IgA) Primary and Secondary[+] antibody response to T-dependent and T-independent antigens, B cell enumeration.[+]
Macrophage function	Resident peritoneal cell numbers and nonspecific esterase staining, Phagocytosis Lysosomal enzymes - 5' - nucleotidase, acid phosphatase, leucine amino peptidase Cytostasis of tumor target cells Reticuloendothelial system clearance of foreign material.

(cont'd)

Table 5 (Cont'd)

Parameter	Procedures Performed*
Bone marrow colony forming units	CFU-S-multipotent, hematopoietic stem cells CFU-GM-granulocyte/macrophage progenitors Cellularity ^{59}Iron incorporation in bone marrow and spleen.
Autoimmune response+	Antinuclear antibody determination.

*Abbreviations used are: CBC, complete blood count which includes red blood cell count, hemoglobin, packed cell volume and white blood cell count; CK, creatine kinase; HBD, hydroxybutyrate dehydrogenase; ALT, alinine aminotransferase; BUN, blood urea nitrogen; ACP, acid phosphatase; ALP, alkaline phosphatase; LD, lactate dehydrogenase; CHS, cholinesterase; PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; CFU, colony-forming unit.

+Additions to the list by J. H. Forsell

Modified from Dean et al., 1982.

For procedures, see Luster et al., 1981. and appropriate methods and materials section of this thesis.

tendency in those performing and interpreting the profile results to feel too confident in the results. Especially since the scientific community has approved "this system" as the most appropriate.

It is apparent that systems involved with host defense may be involved in decreasing drug biotransformation in the liver. Perhaps the two systems, drug metabolism of xenobiotics and the recognition of foreign antigens, evolved together. Both are systems which identify, manipulate and help rid the host of "foreign" substances. While the two systems seem to perform the same general functions, one system (immune) deals with molecular weight substances in excess of 10,000 and the other handles smaller molecular weight substances. If, in fact, the two systems are linked by some sort of feedback system, the question becomes why? Is the phenomenon (if real) beneficial to the host, does it work both ways, or is it just a nuisance biological artifact having no discernible benefit to the host?

In order to reduce redundancy, the pertinent literature dealing with pentachlorophenol and the immune system and immunotoxicity in large animals appears in the introductions to the appropriate chapters in this thesis.

Indirect Effects

Whenever immunotoxicology studies are performed, the results must be interpreted carefully. There are a wide variety of physiological, pathological, and environmental factors which can act alone or in combination with other factors to

influence the immune system. Especially important are the hormones of the adenohypophysis and in particular the adrenal glucocorticoid hormones. These hormones can induce lymphopenia and cause depletion of lymphocytes from the thymus and other lymphoid tissues (Dougherty et al., 1964; Claesson and Tjell, 1976). They may also affect the phagocytic activity of macrophages (White and Goldstein, 1972), and cause the sequestration of monocytes as well as their production and release (Van Furth, 1974).

Nutritional factors can also cause immune deficiencies. It is known that children with protein calorie malnutrition have impaired cell-mediated immunity (Schonland et al., 1972); this is also found in rats (Adlard and Smart, 1972). This immunosuppression due to moderate to severe undernutrition may be mediated by increased glucocorticoid levels. However, there are several specific nutritional deficiencies which can lead to decreased host defense. Among these are vitamin B_6 (Robson and Schwarz, 1975) and zinc (Andresen et al., 1973). Natural, additives and inadvertent constituents in food may also cause immunomodulation. Among these are gallic acid (Archer et al., 1977), a food additive carrageenan (Thomson et al., 1976), and bacterial toxins, such as staphylococcal enterotoxins A and B (Smith and Johnson, 1975). Pesticide residues on food can also cause problems. This subject will be discussed later. Minute quantities of an endogenous protein, alpha-fetoprotein (AFP) can cause a variety of noncytotoxic immunosuppressive effects (Murgita and Tomasi, 1975). Normally, AFP is

produced in the yolk sac of the fetal liver and disappears or reaches very low levels after birth. It is believed that AFP is able to protect the fetus from immunological attack by the mother (Mizejewski and Grimley, 1976).

Alpha-fetoprotein becomes elevated in animals with chemically induced liver tumors (Kroes et al., 1975) or animals exposed to hepatocarcinogens (Kroes et al., 1973). When AFP is elevated in post-fetal life, it is often a tumor antigen. Its detection is being used in human medicine to help in diagnosis of hepatomas, germinal cell tumors and other conditions. Apart from these roles, AFP when induced, may then play a significant role in immunosuppression.

Other possible effects on host defense may be mediated by chemicals through mechanisms not yet understood. Because of this, it is important that the researcher not overlook these possibilities and that the appropriate controls be run.

Immunology and Drug Metabolism

Disease therapy involving drugs which depend on cytochrome P-450 for biotransformation and elimination is often complicated by changes in the cytochrome P-450 steady state levels. Changes in drug biotransformation due to enzyme induction or inhibition account for a large number of observed drug reactions and interactions (Goldstein et al., 1974).

Recently, evidence of immunostimulants affecting drug biotransformation by inhibiting the activity of microsomal enzymes have been reported in both animals and man. (Mosedale and Smith, 1975, Farquhar et al., 1976; Soyka et al., 1976).

If these events prove to be more than idiosyncratic reactions, physicians will have to be concerned about possible interactions between immunotherapeutic agents and other drugs. Previously, immunopharmacology dealt only with the study of the effects of drugs on the immune system and the mechanisms of action of a myriad of endogenous substances capable of modulating the immune response.

The most often reported effect of various immunostimulants on the drug metabolizing system has been depression of biotransformation activity. Animal studies have shown that Bacillus Calmette-Guerin (BCG) (Farquhar et al., 1976), as well as Corynebacterium parvum (Soyka et al., 1976), both stimulants of the cell-mediated immune system, can inhibit the activity of several liver microsomal enzymes.

In man, mean antipyrine serum elimination time (t 1/2) increases from 11 to 14.4 hours five to seven days after patients with metastatic malignant melanoma had received C. parvum i.v. daily for ten days (Rios et al., 1977). Also, drug biotransformation is decreased when reticuloendothelial cell activity is either activated or depressed (Barnes et al., 1979). When reticuloendothelial system (RES) cells of the liver are loaded with carbon particles, the metabolism and hepatotoxicity of carbon tetrachloride (a chemical which needs to be activated to cause hepatic damage) is decreased (Stenger et al., 1969). Heme-oxygenase could be responsible for this phenomenon, since it can degrade the heme from cytochrome P-450 and is induced in liver reticuloendothelial cells following

treatment with zymosan (Tenhunen et al., 1970) or endotoxin (Gemsa et al., 1974). Both of these agents are known to decrease drug biotransformation (Gorodisches et al., 1976).

A mechanistic explanation for the decreased capacity of experimental animals to metabolize drugs following pretreatment with immunostimulants is yet to be provided. However, assuming that this phenomenon can occur in man, an immunomodulator-induced depression of drug metabolism could result in raised serum concentrations of any concomitantly administered drugs which are primarily dependent on biotransformation in the liver for detoxification or for elimination. In the combined immunotherapeutic and chemotherapeutic approach to the treatment of malignant disease, where many anti-cancer agents are pro-drugs which must be metabolized into therapeutically active compounds, immunotherapy-induced depression of drug biotransformation might weaken the effectiveness of the cancer chemotherapy.

A cell-mediated hypothesis for inhibition of drug biotransformation was based primarily on a remarkable hepatosplenomegaly in mice caused by C. parvum. Mice treated with doses of C. parvum sufficient to cause hepatosplenomegaly were abnormally sensitive to pentobarbitol (Castro, 1974). They also had prolonged anesthesia after pentobarbital or tribromoethanol, but not diethyl ether, suggesting that the mechanism was interference with detoxification (Mosedale and Smith, 1975). This phenomenon was confirmed in rats by Schroeder in 1976. On histologic examination, evidence of the development of microscopic foci of mononuclear cells (not Kupffer cells)

adjacent to injured or necrotic hepatocytes was seen. The mononuclear cell aggregates became very evident five days after C. parvum administration and at day seven were small islands of cells. At this time, some hepatocytes were frankly necrotic, liver weight reached its peak, and MFO activity was at its lowest. An inverse relationship existed between the extent of splenomegaly and the degree of inhibition of MFO activity (Soyka, 1981).

The accumulation of mononuclear cells at a site of necrosis in the body is normal. However, when a splenectomy was performed prior to C. parvum administration, it blocked the effect of C. parvum on drug metabolism in the liver. The same effect was observed when whole body irradiation was used instead of splenectomy. The only difference between the two procedures was that splenectomy must be done prior to C. parvum administration while irradiation would work concomitantly or 24 hours after C. parvum administration. This suggests that cells of the RES or circulating immune cells elsewhere in the body can inhibit MFO activity. Involvement of the RES, particularly the spleen, indicates that at least some of the effects described are not simply due to liver injury.

It is becoming increasingly apparent that most immunomodulators effectively decrease cytochrome P-450 levels and the liver's capacity to metabolize drugs. This raises questions about the status of drug biotransformations during natural stimulation of the immune system by viral infections.

A decrease in hexobarbital oxidase has

been seen in murine hepatitis and in man, hepatitis infections have been observed to decrease drug metabolism (Rowland et al., 1976). An interesting and relevant example of this interaction in man involves the drug theophylline. Patients getting 200 mg oxtriphylline every six hours (equivalent to 128 mg of theophylline) for seven days showed evelated serum levels of theophylline with 12-24 hours of vaccination with influenza vaccine. In one patient, theophylline levels in the blood increased to the toxic range and were accompanied by several symptoms associated with theophylline toxicity. The duration of this effect is unknown, however the theophylline levels remained high for at least 72 hours, at which time theophylline administration was stopped because two patients were showing signs of a toxic reaction.

The elimination rate of a single oral dose of theophylline was also significantly decreased in four healthy volunteers 24 hours after administration of influenza vaccine. The t 1/2 changed from 3.3 hours to 7.3 hours (Renton et al., 1980). This phenomenon was also reported by Chang et al., in 1978. Renton (1978) also noted the decreased elimination of theophylline in children during acute viral infections of the upper respiratory tract. The suggested cause was decreased hepatic biotransformation of theophylline by the mixed-function oxidase, P-450 system during the acute phase of the viral infection. No dietary restrictions were imposed during these human studies, plus many other factors are known to alter theophylline clearance (Ogilvie, 1978). However, conditions in each patient appeared identical both before and after vaccination.

This is a controversial area because not all research supports an interaction between the immune system and the MFO system. Mice given C. parvum and phenytoin (which undergoes extensive metabolism), show no alteration in phenytoin metabolism as measured by t 1/2 or AUC (area under the serum phenytoin concentration - time curve). This occurred even though the liver weight was increased and the P-450 activity (expressed either as nMoles cytochrome P-450/mg protein or as nMoles cytochrome P-450/g tissue), was significantly decreased (Mullen, 1981).

When phenytoin was given to eight human volunteers (four healthy and four with disseminated malignancy, but all having normal liver profiles), both before and after immunotherapeutic regimes of either BCG or C. parvum, no difference was seen for AUC, Km and Vmax. Also, no effect was seen on the urinary excretion of 5-(p-hydroxyphenyl) - 5 - phenylhydantoin, the major metabolite of phenytoin. However, the cancer patients as a group had significantly lower values for AUC and Km when compared to the healthy volunteer subjects (Wan et al., 1979).

Antipyrine, a drug not affected by serum proteins (as phenytoin is) was also unaffected by C. parvum pretreatment in cancer patients (Hamilton et al., 1980). Also, no changes in MFO activity were found in ducks having hepatitis, nor in some cases of hepatitis in humans, in which drug metabolism remained unchanged or is actually increased (Rowland, et al., 1976).

The previous studies indicate that the relationship between MFO activity and immunostimulation of cell-mediated immunity and RES does not always exist. This statement must be tempered because these studies used single therapeutic doses of the immunostimulants, and perhaps repeated doses are required in some cases to observe these interactions. Also,only certain receptors and metabolic pathways may be affected by immunostimulation. This would result in a "drug-specific" response, or "immunostimulant-specific" response or combination of both. Another concern is the use of cancer patients in some of the studies, who could have altered biotransformation capacity.

Other substances which were found to decrease cytochrome P-450 levels (Renton, 1981) included fungal products (statalon), a virus (Mengo), lipopolysaccharide (E. coli endotoxin), double stranded nucleotides (poly rI. rC), malarial infections (Plasmodium berghi) (McCarthy et al., 1970), tilorone (Renton and Mannering, 1976), and bacteria (pertussis vaccine) (Renton, 1981). One common feature of all these biologicals was the ability of each to induce interferon. Perhaps interferon production is linked somehow to drug metabolism.

Tilorone, an antiviral agent thought to stimulate interferon synthesis (Renton and Mannering, 1976), decreases P-450 in rats and mice. The effect is not immediate and no endoplasmic reticulum damage is apparent. Tilorone also doubled hexobarbital sleeping time and greatly diminished hexobarbital blood clearance rate in rats. Thus, tilorone has a depressive

effect on hepatic drug-metabolizing enzymes in vivo (Renton and Mannering, 1976).

Regardless of the dose of tilorone used, no greater than 40 - 50% of P-450 activity was lost. This suggests either that certain of the P-450 cytochromes are not vulnerable to the action of tilorone or that tilorone evokes a new steady-state level of cytochrome P-450, either by increasing its degradation or depressing its synthesis. This last situation is unlikely because the rate of incorporation of δ-amino-levulinic acid and heme into P-450 was not affected by tilorone (Renton and Mannering, 1976).

If tilorone produces a lowered steady-state level in the hepatic mono-oxygenase system, it is possible that it may affect the same site responsible for the maintenance of the system under normal conditions, and that this site may be affected in the opposite manner by inducing agents such as phenobarbital. Phenobarbital and other inducing agents have enabled us to increase the activity of the hepatic mono-oxygenase system; perhaps tilorone will permit us to lower the activity of this system at will.

Renton (1981) has proposed that the ability to depress cytochrome P-450-dependent mono-oxygenase is a property of all interferon-inducing agents and may be related to the production of interferon itself. However, interferon-inducing agents affect more than one aspect of host defense, and biologic effects (other than antiviral) can be attributed to "contaminating" substances (lymphokines or other mediators of

cellular immunity) found in the crude or partially purified preparations used in experiments (Sonnefeld et al., 1980).

Renton (1981), showed that dextran, dextran sulphate and latex beads, all of which can be phagocytosed by RES cells in the liver, also decrease cytochrome P-450 and related drug biotransformation. None of these agents stimulates the production of interferon. It is also curious that poly rI.rC, an interferon inducing agent, has no effect on drug biotransformation in mice immunosuppressed with cyclophosphamide or in homozygous nu/nu nude athymic mice (Renton, 1981). Since interferon production has been reported in both cyclophosphamide-treated mice and in nude mice, a decreased P-450 may be related to other aspects of the immune system rather than interferon production.

Two broad categories of human interferon have been identified on the basis of physical properties and type of stimulus needed for induction (Valle et al., 1975). One interferon,designated as type I or "classical" interferon, is produced in vitro by buffy coat leucocytes or by non-lymphoid cells in response to virus and is stable at low pH (pH 2) and to heat (1 hour at 56^{o}C). Recently this interferon has been divided into two distinct types, "alpha" and "beta" (Baron, 1979). The other interferon, type II, is now called "gamma" immune interferon,a mediator of cellular immunity, and is produced in vitro by lymphocytes in response to mitogens or specific antigens, and is acid and heat labile.

All of the interferon inducers to date, including

tiloronehydrochloride and polyriboinosinic-polyribocytidylic acid, are potent inducers of alpha and beta interferon. It is the gamma interferon, however, which has been shown to be significantly more potent (as much as 100 times more potent) in regulation of immune response (Sonnefeld et al., 1980).

When passive transfer of exogenous gamma interferon preparations was performed in mice, a significantly depressed aminopyrine N-demethylase and cytochrome P-450 resulted. Passive transfer of "mock interferon" (produced by inoculation of tuberculin into nonsensitized mice), normal serum, or equivalent amounts of type I interferon had no effect (Sonnenfeld et al, 1980). This observation may account for the varied findings in previously mentioned studies. The effects of interferon on the cytochrome P-450 system may not be a direct cause and effect relationship. It has been shown that interferon has several activities that are not directly antiviral (Gresser, 1977). Among these are inhibition of cell division of both tumor and normal cells, and enhancement of cell function (i.e., phagocytosis by macrophages, cytotoxicity for tumor target cells by sensitized lymphocytes, increased number of antibody-forming cells, and production of proteins including interferon by "priming"). An interesting example of protein priming is the increased amount of a specific enzyme, aryl hydrocarbon hydroxylase,synthesized in interferon-treated cells after induction with benzanthracene (Nebert and Friedman, 1973). One other type of interferon activity not classified as directly antiviral is its effect on cell surfaces. Interferon can drastically change the number of surface

histocompatibility (H-2) antigens or cause a marked accumulation of viral antigens at cell surfaces of virus-injected murine lymphocytes (Friedman et al., 1975; Pitha et al., 1976). Until the effects of interferon are determined, researchers should be prepared to accept that interferon-treated cells may display activities other than antiviral which may be involved in drug metabolism.

The amount of interferon produced appears to be under genetic control. DeMaeyer and DeMaeyer-Guignard (1980), described IF loci which direct either high or low interferon production when mice are exposed to different viruses. When challenged with Newcastle Disease Virus (NDV), only a single autosomal locus (IF-1) responsible for interferon production was identified and this contained two alleles, "h" for high and "l" for low interferon production. In response to other viruses, at least three other separate loci could be identified.

In mice containing the ^{h} allele at IF-1 cytocyrome, P-450 was decreased by 35% 24 hours following injection of NDV. In another strain of mice containing the l allele at IF-1, cytochrome P-450 was not significantly changed compared to controls. This indicates that interferon production at the IF-1 locus causes a decrease in cytochrome P-450.

The experiments with gamma interferon and with inbred strains of mice indicate that only certain types of interferon, or interferon produced on certain gene loci, can lead to a decrease in drug biotransformation. This decreased drug biotransformation due to an "interferon" mechanism would not preclude

decreases caused by other mechanisms operating during the defense of a host against an invading organism.

One of the interesting aspects of interferon is the variety of infectious agents and chemical substances capable of inducing its synthesis. As with other mediators, there is the suggestion that interferon production is the cells' response to an infectious agent or chemical that either disturbs the cell surface and/or stimulates cell division.

Immunosuppressive Chemicals

Drugs

Most immunosuppressive chemicals are drugs. Most of these drugs were developed as antineoplastic agents. Because of their mode of action, they are not selective against cancerous tissue, affecting all rapidly dividing cells they are able to contact in sufficient quantity, including hematopoietic cells and lymphocytes. There are many reviews concerning drugs that are immunosuppressive (Hersh, 1974; Bach, 1975; Zschiesche, 1975), so these will not be discussed here. In view of the large number and diverse structure of immunosuppressive drugs, it is not surprising that many immunosuppressive chemicals exist in our environment.

TCDD

The compound 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and related congeners and isomers are highly toxic impurities present in some pesticides such as 2, 4, 5-trichlorophenoxyacetic acid or 2, 4, 5-T (Elvidge, 1971). These chemicals are also produced as byproducts in a number of chemical processes

and in combustion (Woolson et al., 1972). TCDD was toxic to all mammalian species investigated. The precise effects often depend on the species studied with the guinea pig being the most sensitive. However, a consistent effect of TCDD is damage to the thymus gland, manifested by cortical atrophy and thymic involution. As might be expected with thymic atrophy, cell-mediated immunity appears to be primarily effected by TCDD exposure. Table 6 summarized representative studies covering the range of immunosuppressive effects of TCDD in laboratory animals.

Newborns' cell-mediated immunity can be suppressed by treatment of their mothers with TCDD during gestation and nursing (Vos and Moore, 1974). The suppression caused by TCDD in mice (10 ug/kg, single dose) is reversible (Sharma et al., 1979). All immune systems monitored in this study recovered by eight weeks post-TCDD, including partial recovery by the thymus. This recovery occurred while hepatic lesions persisted. In addition, the effects of TCDD were not prevented by adrenalectomy (Van Logten et al., 1980), indicating that TCDD effects are not mediated through this system.

In humans, no known immunosuppressive effects of the types mentioned above have been discovered. Most human TCDD exposure information is a result of exposure due to the industrial accident in Seveso, Italy in 1976 (Walsh, 1977). A reactor used for the manufacture of trichlorophenol exploded and contaminated 400 acres and its residents. Other TCDD data is from studies on exposure of Vietnam soldiers and

Table 6

Selected Immunosuppressive Effects of TCDD in Various Laboratory Species

Species	In vivo or In vitro Treatment	Observed Effect	Representative Reference
Guinea pigs	in vivo	-thymus atrophy	Vos et al., 1973
"	"	-decreased lymphocyte count	"
"	"	-decreased delayed-type hypersensitivity	"
"	"	-decreased antibody response to tetanus toxoid	"
Mice	in vivo	-thymus atrophy,decreased lymphocyte count	Vos et al., 1973
"	"	-reduced graft vs host activity	"
"	"	-reduced rejection of skin allografts	Vos and Moore, 1974
"	"	-reduced lymphocyte transformation by PHA	"
"	"	-reduction in serum globulins	Vos et al., 1974
"	"	-increased susceptibility to Salmonella bern infection	Thigpen et al., 1975
"	"	-increased sensitivity to Escherichia coli endotoxin	Vos et al., 1978
"	"	-reduced antitetanus titers	Sharma, 1981
"	"	-reduced antisheep erythrocyte titer	Hinsdill et al., 1980
"	"	-decreased contact sensitivity to dinitrofluorobenzene	"
"	"	-reduced resistance to Listeria monocytogenes	"
"	"	-reduced antibody response to TNP-Brucella abortus	Clark et al., 1981
"	in vitro	-increased suppressor cell activity	"
Rats	in vivo	-thymus atrophy	Vos et al., 1973
"	"	-decreased delayed-type hypersensitivity	"
"	"	-reduced graft vs host activity	Vos and Moore, 1974
"	"	-reduced rejection of skin allografts	"

(cont'd)

Table 6 (cont'd)

Species	In vivo or In vitro Treatment	Observed Effect	Representative Reference
Rats	in vivo	-reduced lymphocyte transformation by PHA and Con A	Vos and Moore, 1974
Rabbits	in vivo	-reduced antitetanus titers	Sharma, 1981
"	"	-reduced skin reactivity	"
"	"	-decreased plasma cells in lymph nodes	"

civilians to 2, 4, 5-T, (agent orange and agent purple) used as a jungle defoliant (Baughman and Meselson, 1973). Investigations into these and other incidents of human exposure to TCDD are ongoing.

It now appears that the Ah (aromatic hydrocarbon) gene complex in mice is involved in some of the immunotoxic responses observed in this species (Silkworth and Grabstein, 1982). The Ah gene complex was discovered after aromatic hydrocarbons were found to induce drug-metabolizing enzymes in C57BL/6 (Ah+) mice, but not in DBA/Z (Ah-) mice (Nerbert et al., 1972; Goujon et al., 1972; Nebert and Gilen, 1972; Robinson et al., 1974). Because of these findings (Poland et al., 1974; Nebert et al., 1975), the presence of a cytosolic receptor or receptors controlled by the Ah complex which specifically binds some polycyclic aromatic hydrocarbons (PAH) was postulated. Not only is the presence of the cytosolic receptor required to produce toxicity, due to PAH exposure in the mouse, but a structural requirement exists for the polycyclic aromatic hydrocarbon. The relative binding affinities of some halogenated aromatic hydrocarbons including TCDD is closely correlated with mixed function oxidase induction and the toxic responses observed (Poland and Glover, 1977; Poland and Glover, 1980). The planarity, size and shape of the PAH seem to be important for receptor binding. In C57BL/6 mice the planar molecules TCDD as well as 3, 4, 5, 3', 4', 5'-hexabromobiphenyl bind strongly to the cytosolic receptor and produce thymic involution (Poland and Glover, 1980), whereas the nonplanar

2, 4, 5, 2', 4', 5'-hexabromobiphenyl does not bind to the receptor and does not cause thymic involution. However, the relationship between enzyme induction and toxicity is unclear.

Pesticides

Many pesticides are potentially toxic in man. However, the frequency of systemic poisoning or of other major chronic diseases resulting from pesticides is rare. This illustrates the effectiveness of education and legislation on public awareness in the handling and use of pesticides.

Epidemiologic investigations presently indicate that effects on the immune system are probably the most frequent toxic manifestation of contact with pesticides at infrequent, low level, or chronic exposures. Pesticides usually affect the immune system by eliciting an allergic response. The most common allergic response observed is allergic dermatitis of the cell-mediated inflammatory-response type. This type of allergic dermatitis involves mast cell degranulation and is characterized by erythema, edema and eczema. Typically, there is no respiratory system involvement and IgE antibodies against pesticides, and serum IgG elevations are rare. Another form of allergic response, photoallergy, has also been reported with esposure to several pesticides, including paraquat and captafol (Nomura et al., 1976; Horiuchi and Ando, 1977; Peoples et al., 1978). A list of pesticides reported to cause allergic dermatitis in humans is given in Table 7. Some pesticides elicit the production of autoantibodies. However, the presence of autoantibodies does not necessarily correlate

Table 7

Pesticides Claimed to Have Caused Allergic Response in Humans

Insecticides	Fungicides	Herbicides
BHC	Benomyl	CDAA
Butyphos	Captan	Chlorothalonil
Cartap	Difolatan	2, 4-D
Chlorfenvinphos	Dinofen	Daconil
Chlorobenzilate	Mancozeb	Paraquat
Chlorophos	Maneb	2, 4, 5-T
Cyanox	Nematin	Trifluralin
Cyanphenphos	PCNB	
DDT	Thiram	
Diazinon	Zineb	Rodenticides
Dichlorvos		
Dicofol		Phosphine
Dimite		
Dinofen		
Fenitrothion		
Formothion		
Karathane		
Leptophos		
Lindane		
Malathion		
Methomyl		
Methyl, mercaptophos		
Naled		
Nicutine sulfate		
Nitrofen		
Omite		
Ovex		
Phenthoate		
Pirimiphus-methyl		
Salithion		
Schradan		
Thiometon		
Thiophanate		

with autoimmune disease. Many people have detectable autoantibodies but are asymptomatic.

The types of autoantibodies detected and believed to be pesticide-related are liver, red blood cell, thyroid, kidney, stomach, and intestine. The pesticides reportedly responsible for the development of autoantibodies are DDT, lindane, fenthion, 2, 4-D (Katsenovich and Usmanova, 1970), caprolactam, methenilene-diamine, polychloropinene, thiram, chlorophos (Brusilouski et al., 1973), anthio, milbex (Aripdzhanov, 1973), hexachloroeyclohexane, methylmercaptophos (Omirov and Talan, 1970), phosphamide, aldrin, monuron (Nikolaev et al., 1970), and carbon tetrachloride (Dodson et al., 1965).

Many pesticides have been shown to cause immunomodulation of both cell-mediated immunity and humoral immunity. An extensive list of pesticides and their effects on laboratory animals is given by Sharma (1981). Upon review of this list, it becomes apparent that the present understanding of immunomodulation due to pesticide exposure is largely at the descriptive level. Little or no research has been directed to the question of the mechanisms of immunotoxicity.

In a few pesticides studied, one would question whether immunosuppression occurs at all, or only as an indirect effect. DDT is an appropriate example. In studies of immunosuppression after DDT exposure, often no immunological response was observed unless relatively high levels of DDT were given. These high DDT exposure levels resulted in other toxic effects and death of some exposed animals (Wassermann et al, 1971).

One interesting finding associated with DDT is its apparent ability to protect against anaphylactic shock. Evidently, guinea pigs pre-treated with DDT show less severe episodes of anaphylactic shock (Gabliks et al., 1973). The mechanism for the shock protection appears to be due to the ability of DDT to decrease the amount of histamine available for release at the time shock is induced. DDT does this by inducing the release of histamine from mast cells or by reducing mast cell number (Askari and Gabliks, 1973).

Heavy Metals (Lead, Cadmium, Arsenicals, and Organotin)

The immunotoxicity of metals is best known. Selye et al. in 1966 observed that lead greatly increased the susceptibility of rats to bacterial endotoxin. Selye showed that the damage done in rats by 100 ug of Escherichia coli endotoxin could be reproduced by injecting one ng endotoxin into rats given a normally tolerated dose of lead intravenously (5 mg/100 g body weight) as lead acetate. The change observed represents a 100,000 fold increase in endotoxin sensitivity. Subsequently, this phenomenon has been repeated in mice (Rippe and Berry, 1973) and chickens (Truscott, 1970). Another metal, cadmium, is also able to induce similar changes in rats exposed to Salmonella enteriditis endotoxin (Cook et al., 1974).

Lead and cadmium affect other systems of host defense. Lead decreased host resistance to viral infections (Gainer, 1974). It appears this is due to reduced interferon synthesis. Other studies have shown some decrease in humoral immune response (Koller, 1973) and in phagocytic activity (Trejo et al., 1972;

Filkins and Buchanan, 1973) due to lead exposure. T lymphocyte function is altered by a single exposure to lead (10 mg/kg i.p.) in mice. Antibody response to T lymphocyte-dependent antigens is markedly depressed due to this treatment, but returns to normal in three weeks (Winchurch and Thomas, 1983). Meanwhile, the T independent antigen response was unaffected or raised in this study. The B cell mitogen response to LPS was increased after lead exposure and decreased linearly, falling to control values by three weeks, essentially mirroring the drop in blood lead level in the mice. Follow-up studies in vitro showed that T cells derived from mice treated with lead do not cooperate with normal B cells but that B cells from exposed mice can make antibody when mixed with normal T cells.

The form in which lead or cadmium is used in host-defense studies makes little or no difference in the results obtained (Dr. Pierluigi Bigazzi, personnel communication). How, when, and how often the metal is administered is important (Vos, 1977).

Arsenicals appear to affect host defense by increasing susceptibility to viral infection (Gainer and Pry, 1972). A possible explanation for this is the inhibition of both interferon synthesis and action seen with high level exposure to arsenicals. At low levels of arsenical exposure, the activity of interferon was actually increased (Gainer, 1972). Biphasic responses to xenobiotics are not uncommon in toxicology. Perhaps the beneficial growth-promoting effects seen when arsenicals are used as feed additives is through a reduction in disease which may be in part caused through the increased interferon activity.

Organotin compounds, especially DOTC (di-n-octyltin-dichloride) and DBTC (di-n-butyltindichloride) are very selective immunosuppressants. These chemicals can exert a selective cytotoxic action on T lymphocytes (Seinen and Willems, 1976; Seinen et al., 1977a; Seinen et al., 1977b; Seinen et al.,1979),which results in a T cell dependent immunity depression and an atrophy of the lymphoid system. In fact, this immune suppression is the most sensitive parameter of any toxicity associated with these two chemicals. This makes DOTC and DBTC two of the few "pure" immunotoxicants identified.

The immunotoxicity associated with DOTC and DBTC is more than just a scientific curiosity. It is estimated that more than 25,000 tons of organotins are produced yearly (Van der Kerk, 1978). Among the uses for these chemicals are: as heat stabilizers of polyvinyl chloride plastics; as biocidal compounds for wood, paper, and textile preservation; as an agricultural fungicide, miticide, and acaricide; as a component of antifouling paints; and as catalytic agents in a variety of industrial processes (Ross, 1965; Luyten, 1972). Hence, there are many opportunities for these compounds to enter the environment.

Organochlorines

Polychlorinated biphenyls (PCB) are a class of industrial chemicals which are very stable and have low flammability. They contain from 12 to 68 percent chlorine and are exceptionally persistent in the environment. PCBs have been used for over 40 years in many applications. Among these uses are: as insulating materials in electrical capacitors and transformers, plasticizers,

in waxes; in paper manufacturing; and for a variety of other industrial purposes. The health effects of PCBs are well established. Observed effects in mammals and birds include microsomal enzyme induction, porphyrogenic action, estrogenic activity and immunosuppression (Bitman, 1972; Vos, 1972). There are many good reviews on the effects of PCBs ranging from a consideration of physiochemical properties (Nelson, 1972) to the effects of acute and chronic exposure in laboratory animals (Fishbein, 1974).

PCB seems to cause an immunosuppression which results in an enhanced susceptibility to viral infection and thus appears to have a deleterious effect on cell-mediated immunity. Friend and Trainer (1970) found a slight to moderate suppressive effect due to PCP (Aroclor 1254) exposure in ducks challenged with duck hepatitis virus. In guinea pigs, PCB exposure has resulted in reduced delayed-type hypersensitivity (Vos and Van Driel - Grootonhuis, 1972).

Humoral immunity also appears sensitive to PCBs. Loose and coworkers (1979) showed a decrease in antibody synthesis to the antigen sheep red blood cells, a reduced serum IgA concentration, an increased sensitivity to an endotoxin from Salmonella typhusa, and a decreased resistance to a malaria organism (Plasmodium berghei) in mice. Likewise, Thomas and Hinsdill (1978) showed both a higher mortality rate in mice infected with Salmonella typhimurium and an increased sensitivity to endotoxin. These researchers also found a slight lowering of antibody levels to sheep red blood cells and a reduced gamma-globulin fraction in monkeys. With the exception of the study in

monkeys in which 5 ppm Aroclor 1248 was given in feed for six months, all the above effects resulted from fairly high PCB exposure levels (range of 50-1000 ppm). Thus, some doubt remains as to the effects on the immune system of low level PCB exposure.

Polychlorinated biphenyl-induced immunosuppression could be due to the presence of particular PCB isomers. The isomer 3, 4, 5, 3', 4', 5'-hexachlorobiphenyl was the most toxic of the hexachlorobiphenyls isomers tested in the chicken, mouse (Biocca et al., 1976) and the rat (Vos, 1977), and produced the most severe thymic atrophy in these species. In addition, toxic effects of PCB could be due to chlorinated dibenzofurans present as impurities in some of the PCB preparations (Vos et al., 1970). One of these dibenzofurans, 2, 3, 7, 8-tetrachlorodibenzofuran, has been shown to cause severe thymic atrophy in chickens, guinea pigs, and mice. (Moore et al., 1976).

Recently, Silkworth and Grabstein (1982) have shown that polychlorinated biphenyl immunotoxicity segregates with the Ah gene complex in the mouse. Furthermore, they showed that the immunotoxic potential of PCB is dependent on isomer planarity. These studies were done with 3, 4, 3', 4'-tetrachlorobiphenyl which is planar and gives immunotoxic effects, and 2, 5, 2', 5'-tetrachlorobiphenyl which is nonplaner and does not result in immunotoxicity. The 3, 4, 3', 4'-tetrachlorobiphenyl was found to compete with TCDD for a cytosolic receptor in B6 mice, and like TCDD is a strong aromatic hydrocarbon hydroxylase and cytochrome P-450 inducer (Poland and

Glover, 1977). Hence, PCB toxicity may manifest itself by a mechanism similar to TCDD.

Hexachlorobenzene, like PCB, is also immunosuppressive. In fact, it appears that the actual immune lesions seen are quite similar to those observed with PCB (Loose et al., 1977; Loose et al.,1979).

Polybrominated Biphenyls (PBB)

PBB became important as an environmental contaminant when in 1973 PBB accidentally entered the food chain in Michigan. PBB was a major component in FireMaster FF-1, a commercial flame-retardant chemical. The FireMaster FF-1 was inadvertently substituted for NutriMaster, (magnesium oxide) used as a feed supplement for livestock (Jackson and Halbert, 1974; Dunckel, 1975).

Following this accident, a large number of studies reported PBB-associated immunological effects in cattle and laboratory animals. Among the first were reports that FireMaster BP-6 (FireMaster FF-1 without a 2% anticaking agent, calcium polysilicate added) caused atrophy of the bursa of Fabricius in chickens and depressed antibody responses in guinea pigs (Damstra et al., 1982).

Cattle inadvertently exposed to PBB, and accumulating up to 30 ppm in body fat, showed no altered immune function (Kateley and Bazzell, 1978). In another study involving 114 Holstein cattle, 58 of which had PBB body burdens ranging from 0.02 to 24 ppm for at least two years, no immunomodulation was seen (Kateley et al.,1982). Two additional cattle in the

Kateley study were fed 25 grams of PBB daily for 39 consecutive days. These animals had usual immune competence until they had been fed 500 g of PBB, which corresponded to a tissue concentration exceeding 1,000 ppm. Above 500 grams of PBB fed, the cattle became moribund and exhibited changes in neutrophil function and serum antibody titers.

Beagles fed up to 4.0 mg PBB/kg for 61 days had involuted thymuses and reduced hematopoiesis (Farber et al., 1978). In Rhesus monkeys, alterations in B and T cell functions occurred after feeding PBB at 1.5 ppm for 5 months and 25 ppm for 10 weeks (Allen and Lambrecht, 1978).

PBB has also been reported to affect the immune system in the rat and mouse (Luster et al., 1978; Wilson et al., 1979; Luster et al., 1980). Fraker (1980) showed that antibody responses to sheep red blood cells in mice fed diets containing 1, 10, or 100 ppm PBB for 30 days were 80%, 30%,or 12%, respectively, of control values. Cell-mediated immunity was not affected in this study.

Human epidemiologic studies (Bekesi et al., 1979) have indicated that some farm family members with PBB exposure had exhibited a decreased number of lymphocytes with concomitant increases of lymphocytes with no detectable surface markers, and decreased lymphocyte function as measured by blastogenesis. Bekesi (personal communication) has also indicated that exposed Michigan residents have increased IgG, IgA, and C_3 complement levels as well as increased incidence of neuplasia. Unfortunately, these studies have not been confirmed.

It appears that the dose of PBB required to cause immunosuppression in most animals is very near the limit of overt toxicity. In some animals,no effects were seen (Kateley and Bazzell, 1978; Mudzinski et al., 1979; Kateley et al., 1982), or the animals'response returned to normal, indicating immunological recovery (Wilson et al., 1979). In other studies (Luster et al., 1980),no functional alterations (i.e., bacterial challenge) of the immune system were observed even though several immunological responses were depressed. It appears that the immunotoxicity of PBB, not unlike that of PCB and TCDD, may vary greatly from species to species (Vos, 1977).

Carcinogens

A detailed discussion of carcinogenesis is beyond the scope of this review, however,a few comments should be made since environmental chemicals are believed to be responsible for initiation of most human cancers. For a unified coverage of oncology,see Pitot, (1978).

One of the characteristics of advanced cancer,and especially of neoplasms of the immune system,is a decrease in the host resistance to various infectious agents. Specifically, this phenomenon appears to have at least four characteristics; a decrease in mature granulocytes, an impaired cell-mediated immunity, a decrease or alteration in serum gamma-globulin levels, and production of blocking factors such as "blocking" antibodies and soluble tumor-associated antigens.

Cancer patients are often infected with organisms that normally would not cause disease in the immunocompetent host. This is especially true of fungi and cytomegalovirus, which are ubiquitous in the human population but rarely cause disease in healthy people. So, in a sense, chemical carcinogens as well as radiation can be considered immunotoxicants, since in many cases it is the infectious sequel to the cancer that leads to the ultimate demise of the patient. A partial listing of some known chemical carcinogens is given in Table 8.

Table 8

Examples of Chemical Carcinogens

Benzo(a)pyrene
3-Methylcholanthrene
Benz (a)anthracene
2-Acetylaminofluorene
Pyrrolizidine alkaloids
Aflatoxin B_1
Safrole
3-Hydroxyxanthine
Dimethyl nitrosamine
Acetamide
Thioacetamide
Ethionine
1,2-Dimethylhydrazine
N-Methylnitrosourea
Beta-propiolactone
Dimethyl sulfate
Bis (chloromethyl) ether
Ethylene bromide
Benzyl chloride
Bis (2-Chloroethyl)sulfide
O-Toluidine
Vinyl chloride
Carbon tetrachloride
Thiouracil
Acrylonitrile
Tetrachloroethane

Weisburger and Williams, 1980

ISOLATED HEPATOCYTES

Isolation of Hepatocytes

Berry and Friend (1969) developed the first procedure for the isolation of viable hepatocytes from rats. Their procedure was an *in situ* process using a solution of collagenase and hyaluronidase to separate the hepatocytes. In 1972 and 1973 Seglen published a detailed description of the factors required for optimizing the isolation of rat hepatocytes. Seglen was the first to perform the two-step method of isolation which has become the basis for all subsequent isolation procedures. In the two-step procedure,calcium is removed before enzymatic dispersion using collagenase. This procedure enhances enzymatic dispersion by first breaking desmosomes.

The basic *in vivo* perfusion technique presently used in dissociating rat hepatocytes involves the surgical cannulation of the inferior vena cava. This cannulation is done anterior to the kidneys. The thoracic portion of the inferior vena cava is clamped and the organ is bled out through the portal vein. Calcium-free media is pumped through the liver prior to the addition of the enzyme.

Collagenase is perfused through the liver until the entire organ is visibly softened. The liver is removed, the capsule dissected away, and the cells are mechanically dispersed. The cells are then purified and used as is, or cultured, (See Figure 4).

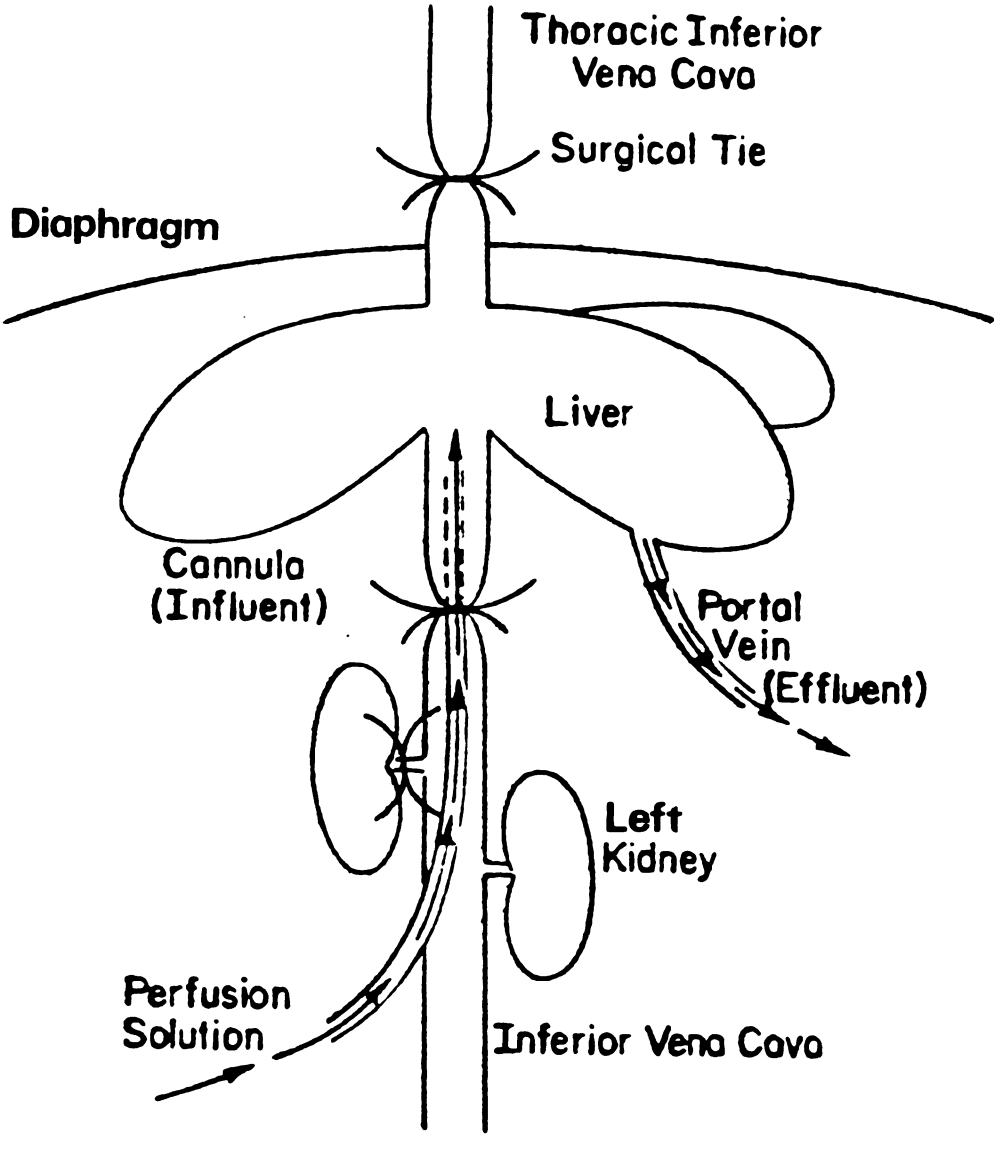


Figure 4. Reverse Perfusion Technique for Rat Liver

Modifications of this system are many. Perhaps the most notable and most used modification is that of Fry et al.,(1976). Fry removes the liver immediately after the rat is killed. The liver is placed in Ca^{2+} and Mg^{2+} free buffer and then cut to obtain liver slices 0.5-1.0 mm in thickness. The slices are then placed in a flask and are shaken in a 37°C water bath. The slices go through a series of calcium-free medias, EGTA-supplemented medias and finally the digestion solution which contains collagenase and hyaluronidase. The flasks are incubated until the supernatant is cloudy. This supernatant containing the isolated cells is filtered to obtain the hepatocytes.

The rat hepatocyte isolation procedure proved to be easy and very effective in obtaining large numbers of viable hepatocytes. A virtual quantitative recovery of viable rat parenchymal cells was readily accomplished by the early 1970's (Seglen, 1973b).

In recent years, isolation of cells from other species has been accomplished. Species in which attempts to isolate hepatocytes has been successful are sheep (Clark et al., 1976; Ash and Pogson, 1977), dogs (Reese and Byard, 1981), monkeys (Poole and Urwin, 1976) and humans (Nau et al., 1978; Reese and Byard, 1981). To accomplish isolation of hepatocytes from these larger species, the procedures described above had to be modified. Most of the modification dealt with the methods used in tissue procurement and initial handling. Surgical remnants, biopsies, liver obtained from aborted fetuses (Nau et al., 1980); Reese and Byard, 1981; Strom et al., 1982) or portions of the liver must be used (Clark et al., 1976).

Isolated Hepatocyte Function

Traditionally, the viability of isolated hepatocytes has been determined by histochemical means. The major method used is trypan blue dye exclusion. Trypan blue exclusion has been and still is being used by almost all researchers requiring a viability determination. However, there is a problem with trypan blue dye exclusion in that it is not very sensitive (Krebs et al., 1979) and is likely to give false positive (viable) results. The procedure cannot distinguish between healthy cells and damaged and denenerating (but still "alive") cells. As a result, many investigators have had to establish viability of isolated cells by means other than trypan blue. Biochemical and histologic evidence of viability have become acceptable means of establishing true cellular viability.

Some of the biochemical tests used have been designed solely to provide information on the cell's viability before they are used in research projects. The more useful viability tests have been discovered secondary to their use in other research.

Gluconeogenesis is a biochemical test used to establish cellular viability. It is especially valuable as an indicator of cellular viability if precursors, biochemically (reactions) far removed are used for its synthesis (Krebs et al,,1979). Another procedure which has proved useful in cellular viability testing is stimulation of cellular respiration by succinate. Only a damaged plasma membrane will allow low levels of succinate to permeate at a rate sufficient to stimulate cellular respiration

(Bauer et al., 1975). Other tests which have been used are: 1) intracellular potassium and adenine nucleotide concentration (Ash and Pogson, 1977); 2) retention of constitutive enzymes like lactate dehydrogenase which are released into the media when membrane damage occurs (Clark et al., 1976);3) electron microscopy of isolated cells (Berry and Friend, 1969); 4) the demonstration of a response to a hormone such as glucagon (Zahlten and Stratman, 1974); and the synthesis of urea (Krebs et al., 1979).

Isolated Hepatocytes and Xenobiotic Metabolism

Isolated hepatocytes have been used for xenobiotic metabolism and cytotoxicity studies. The use of isolated hepatocytes to study xenobiotic metabolism has several advantages over use of the whole animal. Compared to the whole animal, cells are essentially free of hard-to-control variables such as hormone or nutrient levels (Fry and Bridges, 1977). Also, dosing,time of exposure, and metabolite generation can be more rigidly controlled (Fry and Bridges, 1977) and done more inexpensively than in a large animal. Another advantage of cells is that several variables can be tested using a single preparation of cells.

Other alternatives to the whole animal approach are in vitro systems such as organ perfusion, tissue slices, subcellular fractions and tissue maintenance cultures. Although all of these techniques have been successful in some studies, more and more researchers are using isolated cells in xenobiotic metabolism studies simply because the system seems to

mirror the in vivo situation more closely (Fry and Bridges, 1977; Menzer, 1979). Specifically, some of the advantages of the isolated cells system cited by researchers include: dead or deteriorated cells can be selectively removed from the system (Belleman et al., 1977); suspended or cultured cells are totally functional (Nau et al., 1979); isolated cells retain complete subcellular organization (Nau et al., 1978); cells remain viable for several hours (Nau et al., 1978; Nau et al., 1979); and the environment of the cells can be manipulated (Holtzman et al., 1972; Erickson and Holtzman, 1976) so that hormonal or other homeostatic mechanisms can be controlled. In addition, the requirements for optimal metabolic rate can be established (Bissell and Guzelian, 1979); cofactor additions are not required (Holtzman et al., 1972; Nau et al., 1978); and the total metabolism of xenobiotic can be studied (Billings et al., 1977), including the interrelationships between metabolic pathways (Wiebkin et al., 1976; Morello, and Agosin, 1979) and substrates (Hayes and Brendel, 1976). The full sequence of reactions (Moldeus et al., 1978) and the rate limiting steps (Shinkey et al., 1979) can be studied as can the further metabolism of primary metabolites (Wiebkin et al., 1978). In isolated hepatocytes, conjugation pathways are fully functional (Jones et al., 1978) and the depletion of conjugating components like GSH can be monitored (Hirata et al., 1979). Moreover, cells lend themselves to the study of rapid reaction sequences and make quantitation of short-term reactions possible (Moldeus et al., 1974). Xenobiotics which have been studied using isolated hepatocytes are listed in Table 9.

Table 9

Xenobiotics Which Have Been Studied Using an Isolated Hepatocyte System.

Xenobiotic	Reference
alpha-1-acetyl methadol	Billings et al., 1977
propoxyhene	"
butamoxane	"
ethinimate	"
methoxybutamoxane	"
p-nitrophenol	"
ethylmorphine	Erickson and Holtzman, 1976
aminopyrine	Poland and Kappas, 1971
aniline	Morello and Agosin, 1979
benzoic acid	"
phenol	"
hexobarbital	Nau et al., 1978
diphenylhydantoin	"
benza (a) pyrene	Nau et al., 1979
benzodiazepine drugs	"
biphenyl	Wiebkin et al., 1978
ethoxycoumarin	Fry et al., 1976
7-hydroxycoumarin	Moldeus et al., 1977
alprenolol	Moldeus et al., 1974
phenophthalein	Moldeus et al., 1978a
4-methylumbelliferone	"
2-napthol	"
harmine	"
paracetamol	Moldeus et al., 1978b
quinine sulfate	Hayes and Brendel, 1976
dichloro-p-nitrophenol	Hultmark et al., 1978
ethoxyresorufin	Burke and Orrenius, 1978
thiabendazol	Gerayesh-Nejad et al.,1975
chloramphenical	Siliciano et al., 1978
amphetamine	Billings et al., 1978
p-nitranisole	"
sulfadimidine	Morland and Olsen, 1977
sulfanilamide	"
p-aminobenzoic acid	"
cocaine	Stewart et al., 1978
2-acetylaminofluorene	Leffert et al., 1977
napthalene	Block et al., 1976
acetaminophen	Andersson et al., 1978
harmol	"
aflatoxin B_1	Decad et al., 1977
phenyramidol	Jones and Mason, 1978
para-chloro-N-methylaniline	Dougherty et al., 1980

Future of Isolated Hepatocytes

The continued use of isolated hepatocytes in toxicology and pharmacology studies depends on the success of researchers in maintaining cytochrome P-450 levels over extended periods of time. P-450 deteriorates after about 3-5 hours of incubation (Michalopoulos et al., 1976; Decad et al., 1977). Recently, P-450 longevity has been increased by defining nutritional (Bissell and Guzelian, 1979; Paine and Hockin, 1980) and hormonal requirements (Michalopoulos et al., 1976; Decad et al., 1977). The induction of P-450 in culture is very difficult to accomplish and only a few researchers have been able to do so (Sinclair et al, 1979). Higher in vitro levels of cytochrome P-450 are routinely obtained by first inducing in vivo.

The future of isolated hepatocytes may be altered by the discovery of a method for division and multiplication of differentiated hepatocytes. Presently,hepatic function itself is lost in 3-7 days after isolation. The isolated hepatocytes dedifferentiate and lose hepatic function as evidenced by loss of albumin secretion. This dedifferentiation may be responsible for the loss of P-450. Although multiplication of hepatocytes in vitro has not yet been achieved by the scientific community, there is hope that this will occur when all the necessary parameters are defined, because hepatocytes will regenerate in vivo (Bucher, 1963). Maintenance of hepatocytes as differentiated cells has been accomplished for up to 3-5 months by improving support systems (Rojkind, 1980; Freeman, 1981;

and Enat, 1982). If the multiplication of differentiated hepatocytes becomes a reality, isolating hepatocytes will no longer be necessary.

Chapter 1

SUBCHRONIC ADMINISTRATION OF TECHNICAL PENTACHLOROPHENOL TO LACTATING DAIRY CATTLE: IMMUNOTOXICOLOGIC EVALUATION

INTRODUCTION

Technical pentachlorophenol, commonly referred to as "penta", has a variety of commercial applications. Its effectiveness as a broad spectrum biocide is due to the oxidative phosphorylation uncoupling ability of its major component, pentachlorophenol (PCP). It is used commercially as the active ingredient in various molluscicides, herbicides, insecticides, fungicides, bactericides and slimacides. However, most of the penta produced commercially in the Unites States is used for wood preservation.

Since many structural components of livestock facilities such as feed bunks, bunk silos, splash boards, support poles, and free stalls are constructed of penta-treated wood, livestock may be exposed subchronically to preservative chemicals. The extent of usage of treated wood on dairy farms in Michigan was determined by a recent survey which found penta-treated wood on nearly 50% of the farms (Foss et al., 1980). On livestock farms, exposure can result from: 1) ingestion of feeds stored in or fed from penta-treated structures, or licked off of treated wood); 2) cutaneous absorption by direct contact with treated wood; and 3) inhalation of air containing preservative chemicals, particularly the volatile chlorophenols.

An implication of an association between exposure to penta and poor health of dairy cattle was reported by Thomas et al., 1977). Clinical signs in these cattle included decreased milk production, poor general appearance, skin lesions, increased mastitis, persistent infections, high calf mortality,

and death. Certain of these effects suggested a suppression of immune function. Moreover, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (tetra-CDD) causes thymic atrophy in many species (Gupta et al., 1973). Even though penta does not contain 2, 3, 7, 8-tetra CDD, it has been reported to contain a small concentration of other tetra-CDD isomers (Kinzell et al., 1981) as well as hexa-, hepta-and octa-CDD and dibenzofurans (Firestone et al., 1979; McConnell et al., 1980; Kinzell et al., 1981). Various other chlorinated hydrocarbons such as hexachlorobenzene and chlorinated diphenyl ethers (Firestone et al., 1972) also exist in penta. Hexachlorobenzene has been shown to be immunosuppressive in mice (Loose et al., 1977) and rats (Vos et al., 1979), but the immunomodulation of other components in penta has not been reported.

In a study recently reported by McConnell et al. (1980), various defense mechanisms were evaluated in growing dairy heifers fed analytical pentachlorophenol, technical grade pentachlorophenol, or various mixtures of both. The chemicals were administered subchronically at exposure levels 1-2 orders of magnitude greater (20 mg/kg body wt/day for 42 days followed by 15 mg/kg/day for 118 days) than what has been estimated to occur on some dairy farms (Van Gelder, 1977). Toxic effects which included increased mitogen-induced lymphoproliferation and decreased thymus weight were more prevalent in heifers fed technical PCP. Comparatively, the present study, which utilizes a more extensive

immune profile, was designed to ascertain whether a level of exposure approximating a farm environment would modulate the immune system in older lactating dairy cattle. A previous report (Kinzell et al., 1981) contains performance, general health, and pathology findings.

MATERIALS AND METHODS

Chemicals and Reagents

Commercial pentachlorophenol (MB-528) was generously supplied by the American Wood Preservers Institute (AWPI) and represented an industry composite from the three major manufacturers of penta. The chlorophenol composition of this lot as reported by the supplier (Roman, 1978) was 85-90% pentachlorophenol; 4-8% tetrachlorophenols; 0.1% trichlorophenols; and 2-6% other components. The chlorodibenzo-p-dioxins (CDDs) were determined by high pressure liquid chromatography with confirmation by gas chromatography/mass spectrometry. CDD concentrations in ppm were: octa-CDD, 1000; hetpa-CDDs, 378; hexa-CDDs, 173; and tetra-CDDs, 0.035. The tetra-CDD does not include 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. The analytical methodology is described by Kinzell et al., (1981).

Other chemicals and reagents included: Ficoll, Con A and LA, (Pharmacia Fine Chemicals, Piscataway, NJ) and Ca^{2+} and Mg^{2+}-free Hanks balanced salt solution, (HBSS), Hanks balanced salt solution, sheep red blood cells (SRBCs,) fetal calf serum and RPMI 1640 with 25mM Hepes (Microbiological Associates, Walkersville, MD), Vibrio cholerae neuraminidase (VCN), Freunds complete adjuvant, and penicillin and streptomycin (Gibco, Grand Island, NY), fluorescein-labeled (FITC) rabbit anti-bovine Ig reagents and single radial immunodiffusion agarose plates for serum immunoglobulin concentrations (Miles Laboratories Inc., Elkhart, IN), [^{3}H] thymidine ([^{3}H] Tdr), (New England Nuclear Corp., Boston, MA), Hypaque

(or sodium diatrizoate, Sterling Organics, New York), 1.091 micrometer polystyrene latex particles (Dow Chemical Co., Indianapolis, IN), zymosan A (Sigma Chemical Co., St. Louis, MO), BCG (Research Foundation, Chicago, IL) and PPD (Connaught Laboratories, Toronto, Ontario).

Treatment of Cattle

Eight non-pregnant mature Holstein-Friesian cattle, four penta-treated and four controls, were paired according to stage of lactation. During the study, each cow was periodically fitted with an indwelling urethral catheter for urine collection. Also, rumen cannulas were installed in four cows (two pairs) for use in feed digestion trials. The cattle were housed in a cold enclosed barn and allowed a period of five to six weeks for environmental and feed adjustment prior to initiating penta administration. Each pair was started on a treatment 5-7 wks. postpartum. All cattle were milked and fed twice daily. Several additional measurements including serum chemistries, urinalysis and a complete physical examination by a veterinarian were made and reported by Kinzell et al., (1981).

Penta was mixed with the concentrate part of the ration and fed in equal parts twice daily, at the morning and evening feeding times. Treated cattle received 0.2 mg/kg body wt./day for 75-84 days followed by 2.0 mg/kg body wt./day for 56-62 days. Additional details on treatment of cattle were previously reported (Kinzell et al., 1981).

Blood Specimens

Blood for hematological and immunological measurements was periodically collected from each cow by venipuncture. Complete blood counts were performed using EDTA anticoagulated blood and immunological studies were performed with serum, or cells from heparinized blood.

Hematology

Hematological values including red blood cell (RBC) count, hemoglobin (Hgb) content, and packed cell volume (PCV) were determined using a Coulter Counter model S. White blood cell (WBC) differential analyses were performed using Wright's stained blood smears. Erythrocyte sedimentation rates (ESR) were determined using the modified Westergreen method (Dawson, 1960) and a 120 minute end point reading.

Leucocyte Preparations

Lymphocytes and neutrophils were separated from heparinized blood by Ficoll/Hypaque (F/H) gradient centrifugation (Boyum, 1966). A portion of heparinized blood was incubated with carbonyl iron fillings to remove phagocytic cells (phagocyte-depleted leucocytes or PDL) prior to F/H centrifugation. Lymphocytes and neutrophils recovered from the F/H gradient were washed with Ca^{2+} and Mg^{2+} -free Hanks balanced salt solution by centrifugation at 200 x g for 7 min. Remaining erythrocytes were lysed by hypotonic shock using freshly prepared 0.85% NH_4Cl. Following erythrocyte lysis, leucocytes were washed at 350 x g for three minutes with Ca^{2+} and Mg^{2+} -free Hanks balanced salt solution followed by two additional washes at

200 x g for seven minutes in the same solution. For immunologic studies, the cells were resuspended in Hanks balanced salt solution and evaluated for viability using trypan blue dye exclusion. Cell viabilities generally exceeded 95%. Lymphocyte cell suspensions were adjusted to the desired number of cells per ml using a hemocytometer.

Membrane Receptor Assays

Erythrocyte (E) binding (Bach, 1973; Grewal et al., 1976; Higgins and Stack, 1977) was determined by a rosette assay with sheep red blood cells (SRBCs) treated with VCN (Weiner et al., 1973; Reeves and Renshaw, 1978). Two ml of 5% SRBC solution were mixed with 0.4 ml VCN (50 units/ml) for 60 min at 37°C. The VCN treated-SRBC (En) were washed 3 x with Hanks balanced salt solution and adjusted to a 5% solution. For the assay, 0.1 ml of 2 x 10^6 PDL were added to 0.1 ml of 0.5% En in a 10 x 75 mm glass tube and centrifuged at 30 x g for 8 min to form a compact pellet. The cell suspension was incubated at 2-5°C for approximately 16 hr. To enumerate En rosette forming cells (En-RFC), the RBC-PDL pellet was gently resuspended and stained with 0.1% Gentian violet. Lymphocytes with three or more attached En were identified as En-RFC. The percentage of such cell was determined in triplicate by analysis of 200 cells per tube.

Peripheral blood lymphocytes with surface immunoglobulin were enumerated using a polyspecific fluorescein-labeled (FITC) rabbit antibovine Ig reagent (Muscoplat et al., 1974; Reeves and Renshaw, 1978). PDL recovered from F/H gradients were

initially washed with phosphate-buffered saline (PBS) containing 0.1% sodium azide and then incubated with the fluorescent reagent for 35 min at 2-4°C. Following staining, PDL were washed 3 x in PBS and examined microscopically in a wet preparation using a Zeiss ultraviolet microscope. Two to three hundred cells were counted and the percentage of cells with surface bound fluorescence recorded.

Lymphocyte Function Tests

Mitogen-induced blastogenesis of lymphocytes was performed in triplicate using a microculture procedure (Douglas, 1971). Two hundred thousand lymphocytes were cultured in RPMI-1640 media supplemented with 5% fetal calf serum and antibiotics, 100 units/ml penicillin and 100 ug/ml streptomycin, in a microtest tray. Two mitogens, leucoagglutinin and concanavalin A,were used to stimulate the lymphocytes. Blastogenic responses to LA were studied over an 800-fold dose range, while Con A mitogenesis was studied over a 100-fold dose range. The lymphocyte cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere for 72 hr. At 48 hrs, the cultures were pulsed with 25 ul tritiated thymidine($[^3H]$ Tdr) containing 0.25 uCi (specific activity of 6.7 Ci/mM). Cultures were harvested using a multiple analysis sample harvester (MASH-II) and the isotope incorporation evaluated by scintillation counting. The isotope incorporation index (III), the ratio of the radioactivity in the cells stimulated with mitogen to the radioactivity of cells cultured in $[^3H]$ Tdr containing media was determined. In addition, net counts per minute (NCPM)

i.e., the difference between the radioactivity in cells stimulated with mitogen versus control cultures, was determined in cultures responding to optimal concentrations of mitogens.

Serum immunologlobulin concentrations (IgG, IgM, IgA) were determined by radial immunodiffusion (Mancini, 1965; Butler, 1971) using commercial reagents. In addition, during the last month of penta exposure the antibody response was evaluated following intravenous injection of 2 ml of a 10% V/V solution of SRBCs in saline. Serum was collected prior to immunization as well as on days 4, 7, 10, 14, 21, 28 and 35 post-immunization. SRBC agglutinin titers were determined using heat inactivated (56^{o}C for 30 min) serum. The highest serum dilution exhibiting macroscopic agglutination was recorded as the titer.

Delayed type hypersensitivity (DTH) was evaluated *in vivo* by skin testing during the last 30-35 days of the dosing period. Four penta-exposed cattle and three control cattle were injected with Bacillus Calmette-Guérin (BCG) emulsified in Freunds complete adjuvant which was given subcutaneously in the sternal region (brisket). The other control cow served as a DTH testing control and was not given BCG. All 8 cattle were subsequently challenged intradermally 30 days later with purified protein derivative (PPD) and the area of the edematous skin reaction recorded 24 and 48 hours after challenge.

Neutrophil Function Tests

Neutrophil phagocytosis was studied by *in vitro* challenge with 1.091 mm polystyrene latex particles. Neutrophils,

separated from blood on F/H gradients and purified by washings and hypotonic shock, were suspended to approximately 4 x 10^7 cells/ml. Two hundred ul of polymorphonuclear leucocytes (PMNs) were mixed with serum opsonized latex particles and incubated at 37°C for 20 min. The phagocytic reaction was terminated by addition of 0.1 ml glutaraldehyde solution (3%). The PMN suspension was subsequently stained with Turk's solution and examined microscopically. The percentage of PMNs (200 total) phagocytizing at least three particles was determined. Serum for particle opsonization was obtained from penta-treated and control cattle for comparison.

Neutrophil chemiluminescence studies (Allen et al.,1972; Rosen and Klebanoff, 1976; Andersen et al., 1977) was assessed on the day of necropsy. In the assay, 2 ml of PMNs (1 x 10^6 per ml) were added under red light to dark adapted scintillation vials containing 0.5 ml of opsonized zymosan A (S. cerevisia). PMN luminescence was measured at 5 min intervals over a 1 hr period in a Searle Isocap 300 liquid scintillation counter adjusted to detect visible light (non-coincident mode).

Histology

For histologic evaluation the lymphoid tissues were fixed with formalin and stained with hematoxylin and eosin. Special stains used were periodic acid-Schiff (PAS) and Congo Red. A gross and histopathologic examination was performed on each animal and was reported previously (Kinzell et al., 1981). The histological

features of thymus, spleen and lymph node tissue are presented herein.

Statistical Analysis

Statistical analysis was done using SAS (Statistical Analysis System) at Wayne State University, Detroit, Michigan. The data were analyzed using analysis of variance (F distribution) and multiple analysis of variances by the likelihood-ratio test (Gill, 1978). Using this approach,individual points in time were never analyzed individually. Due to the large variation involved in these types of biological tests statistical analyses were designed to examine the change in the difference between paired (penta-treated and control) cattle over time (trend). For some experiments, the Student's t-test for either paired or unpaired data was used.

RESULTS

Hematology

Venous blood, obtained prior to and during the feeding of penta, was evaluated for several hematologic parameters. The erythrocyte counts, hemoglobin concentration, and packed cell volume for control and penta-treated cattle are presented in Figure 1.1. The increases seen in these parameters at the end of the trial are most likely due to hemoconcentration caused by water deprivation prior to euthanasia. Erythrocyte sedimentation rate, which is an indicator of inflammation, ranged from 0 to 0.5 mm/hr throughout the study regardless of penta treatment or occurrence of mastitis. The results show no significant changes in the magnitude of the differences between control and penta-treated cattle over time with respect to these erythrocyte parameters.

The WBC counts for control and penta-treated cattle are presented in Figure 1.2. Again, there were no significant changes in the magnitude of the difference between the penta-fed and control cattle over time. One cow in the penta-treated group became clinically septic with mastitis near the end of the 0.2 mg/kg treatment period. On day 63, the WBC count increased to 24,000 ul. No association between dose and mastitis was made because this particular cow had mastitis before dosing commenced and mastitis occurred in control animals. A second subclinical infectious episode of unknown etiology also occurred in this and another treated cow during the 2.0 mg/kg treatment period. In the first episode, the cow

Figure 1.1. Red Blood Cell Number (RBC x 10^6/ul) Hemoglobin Concentration (HGB g/dl) and Packed Red Blood Cell Volume (PCV%) in Control and Penta-treated Cattle. Each Graphed Point Represents the Mean and Standard Error of all Data Collected Between the Times Indicated. Normal Limits are Those Reported by Duncan and Prasse (1977).

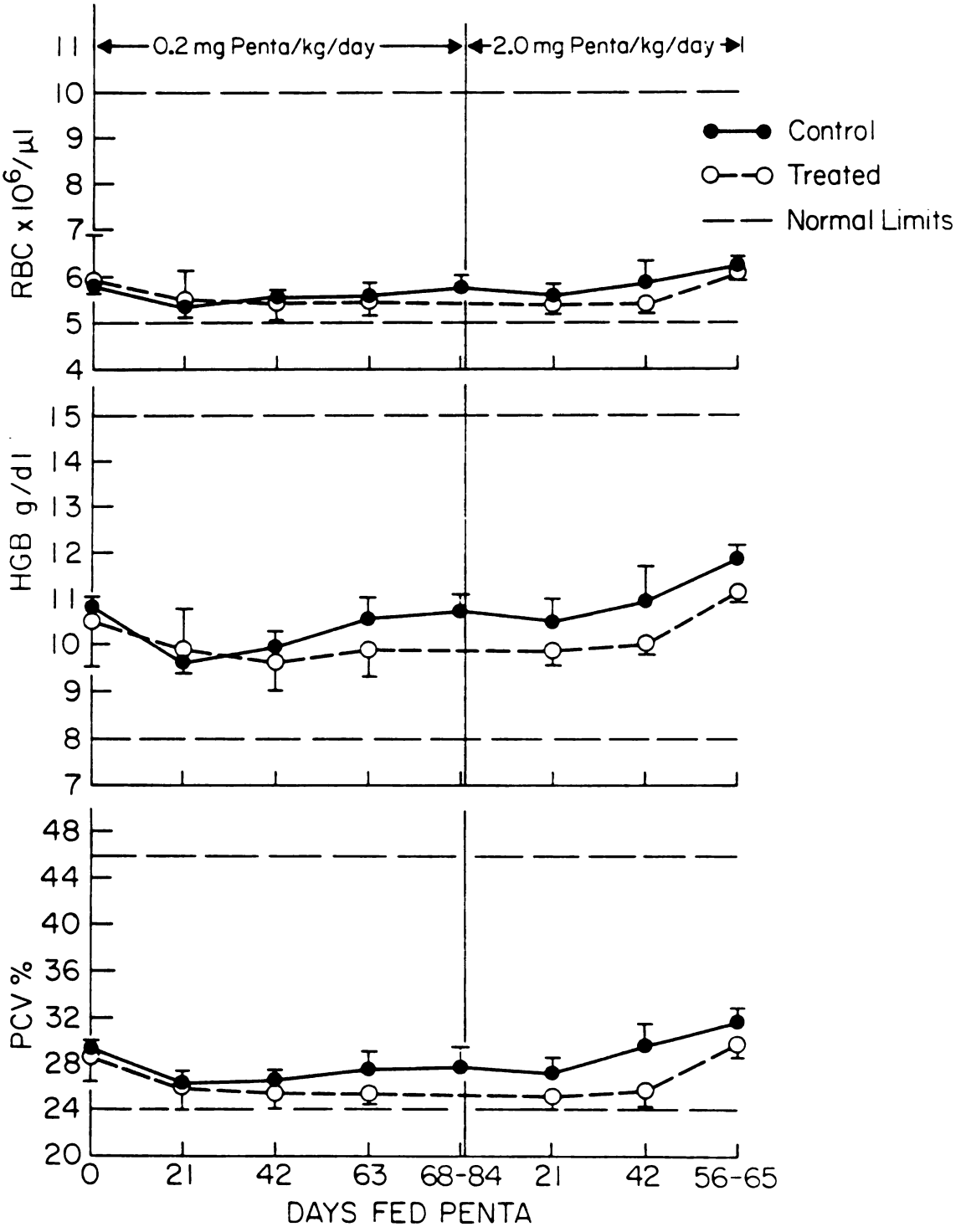
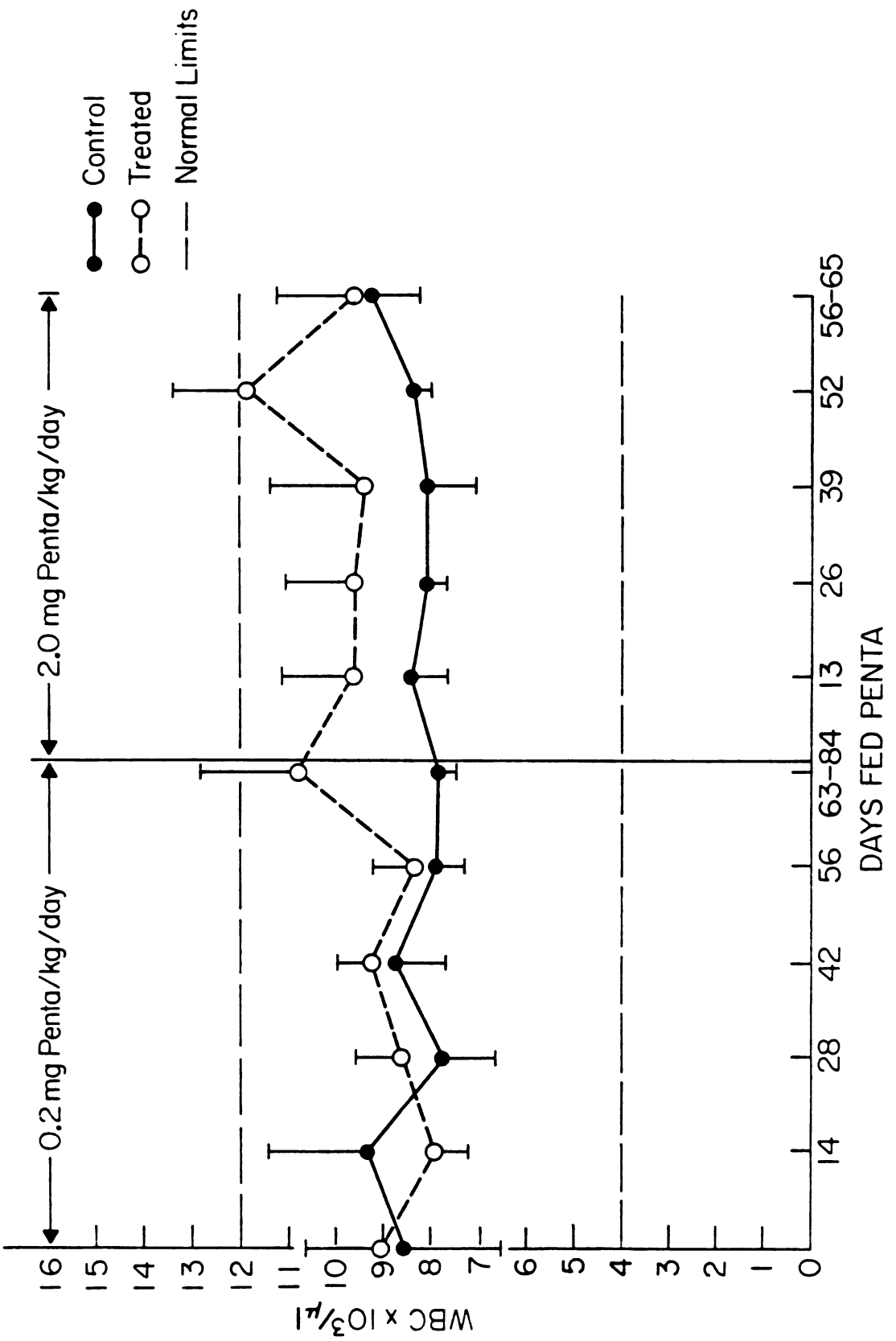

0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
Control
Treated
Normal Limits
RBC x 10⁶/µl
HGB g/dl
PCV %
0
21
42
63
68-84
21
42
56-65
DAYS FED PENTA

Figure 1.2. Number of White Blood Cells (WBC x 10^3/ul) in Control and Penta-treated Cattle. Each Graphed Point Represents the Mean and Standard Error of all Data Collected Between the Times Indicated. Normal Limits are Those Reported by Duncan and Prasse (1977).


Control
Treated
Normal Limits
0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
WBC x 10³/μl
DAYS FED PENTA
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
14
28
42
56
63-84
13
26
39
52
56-65

responsed immediately to antibiotic therapy. In the second episode, the cow recovered without therapeutic assistance.

The numbers of PMNs and lymphocytes in blood of control and penta-treated cattle are presented in Figures 1.3 and 1.4. The magnitude of the difference between penta-treated and control cattle for PMN and lymphocyte counts was not significant when analyzed over time. Moreover, the number of En-RFC and sIg bearing lymphocytes was comparable in the two groups throughout the feeding trial (Figure 1.4).

Lymphocyte Function Tests

In vivo and in vitro immunoassays were conducted to evaluate lymphocyte functions. The blastogenic responses of lymphocytes from control and penta-treated cattle were determined using two T cell-dependent mitogens, LA and Con A (Figure 1.5). The optimal isotope incorporation ($[^3H]$ Tdr)was observed in lymphocyte cultures stimulated with 0.5 ug/culture LA and 0.4 ug/culture Con A. The results indicate that over the course of the study DNA synthesis in penta-treated cattle is neither impaired nor enhanced compared to responses of control cattle. Similar results were also obtained in submitogenic concentrations of the lectins.

Results of skin testing for delayed-type hypersensitivity are shown in Table 1.1. All cattle which were inoculated with BCG and challenged 30 days later by an intracutaneous injection of PPD had a prominent edematous skin reaction within 24 hours. The cow which was not exposed to penta and not sensitized

Figure 1.3. Numbers of Polymorphonuclear Neutrophils (PMN x 10^3/ul) in Control and Penta-treated Cattle. Each Graphed Point Represents the Mean and Standard Error of all Data Collected Between the Times Indicated. Normal Limits are Those Reported by Duncan and Prasse (1977).

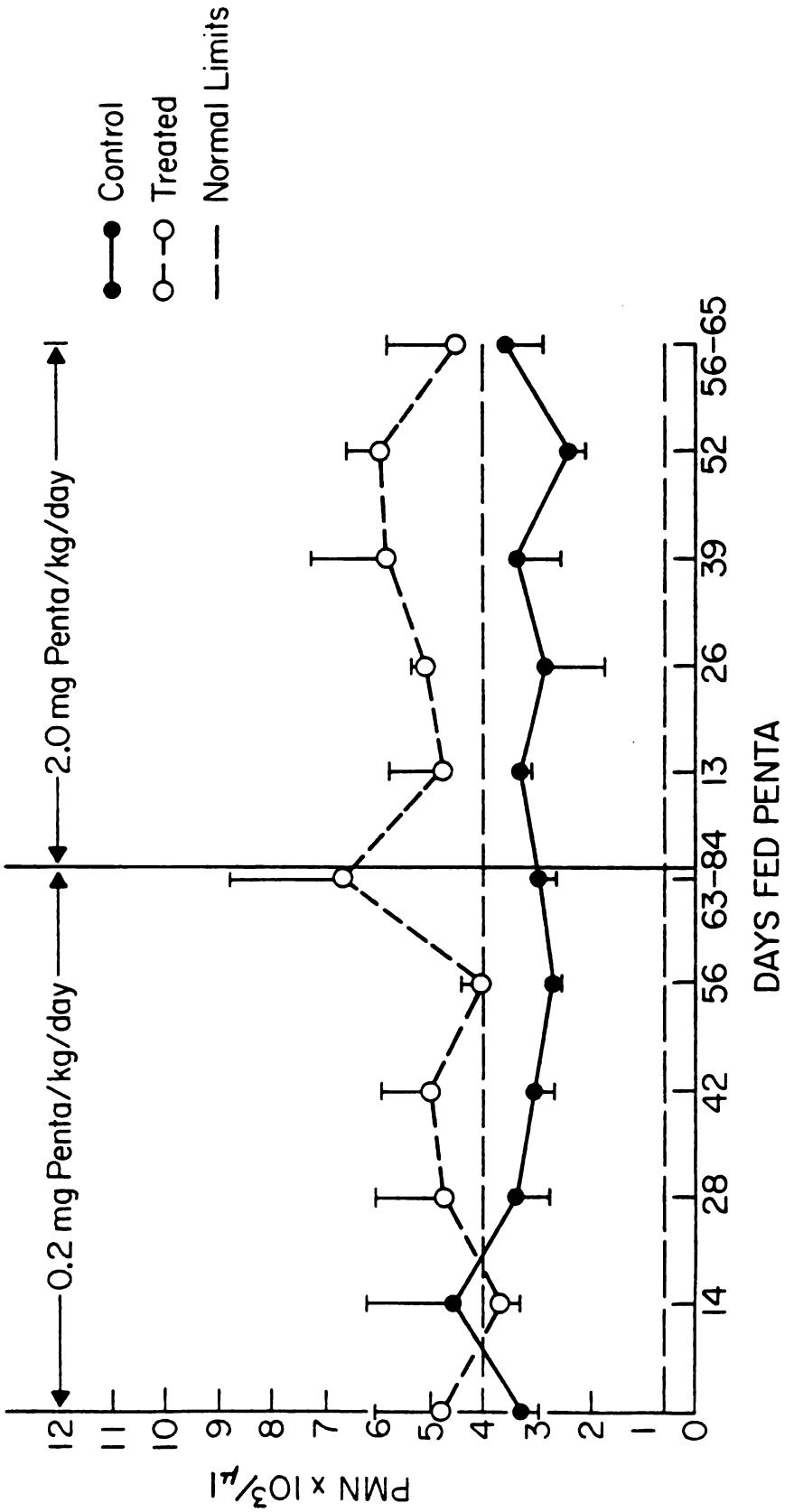

Control
Treated
Normal Limits
0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
PMN x 10³/μl
DAYS FED PENTA
0
1
2
3
4
5
6
7
8
9
10
11
12
14
28
42
56
63-84
13
26
39
52
56-65

Figure 1.4. Total Number of Counted Lymphocytes (mean ± SEM) per ul of Blood and Two Subpopulations of Lymphocytes, 1) Those Possessing Surface Immunoglobulins (sIg) and 2) Those Which Form Rosettes With Neuraminidase-treated SRBCs (En-RFC), in Control (C) and Penta-treated (T) Cattle. The Number of sIg-bearing Lymphocytes is Superimposed Above the Number of En-RFC Lymphocytes; Subtract the Number of En-RFC From the Total of En-RFC and sIg to Derive the Number of sIg Cells. Each Bar Represents the Mean of all Data Collected Between the Times Indicated. The Normal Range of Total Lymphocyte Numbers per ul was Reported by Duncan and Prasse (1977).

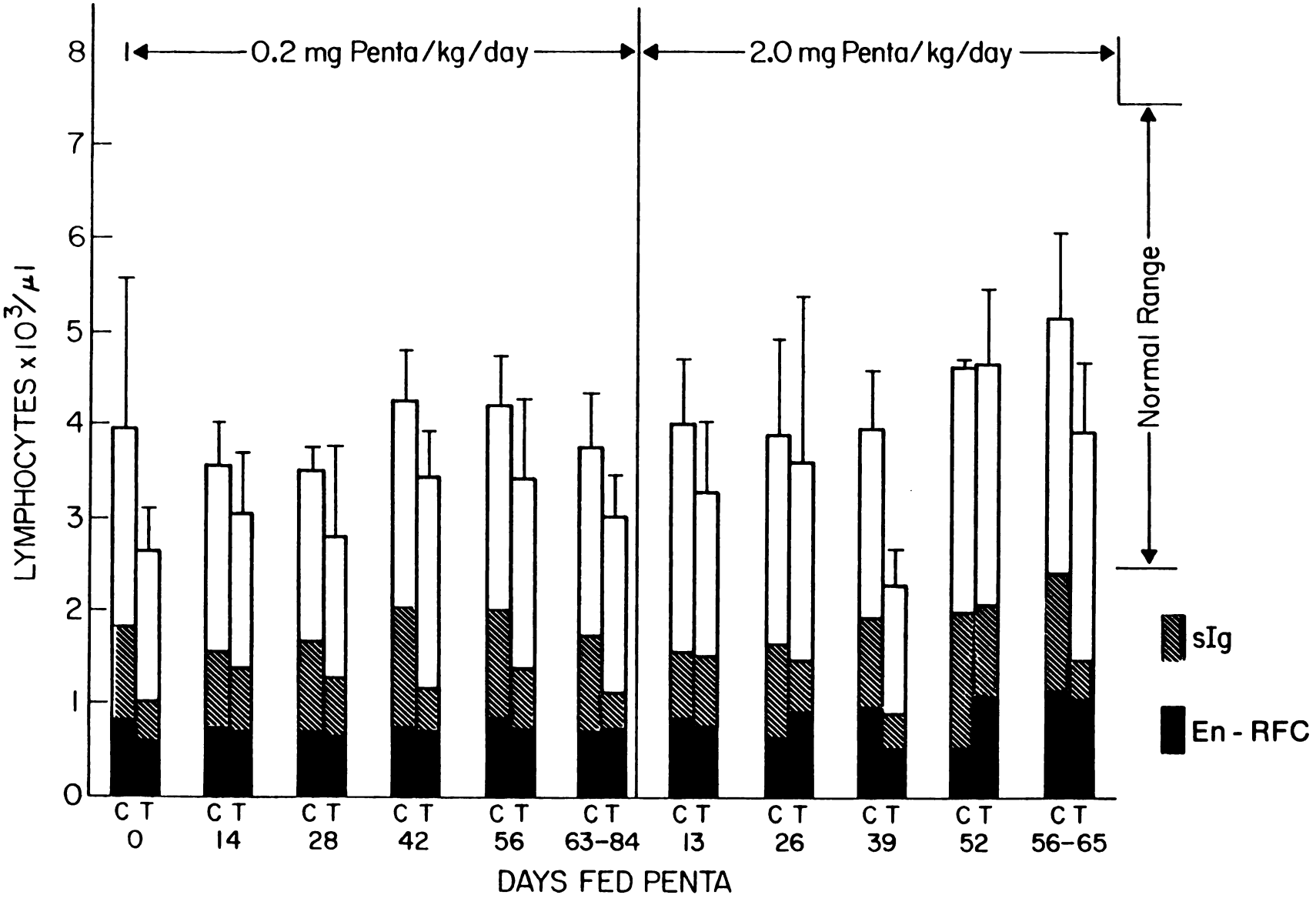
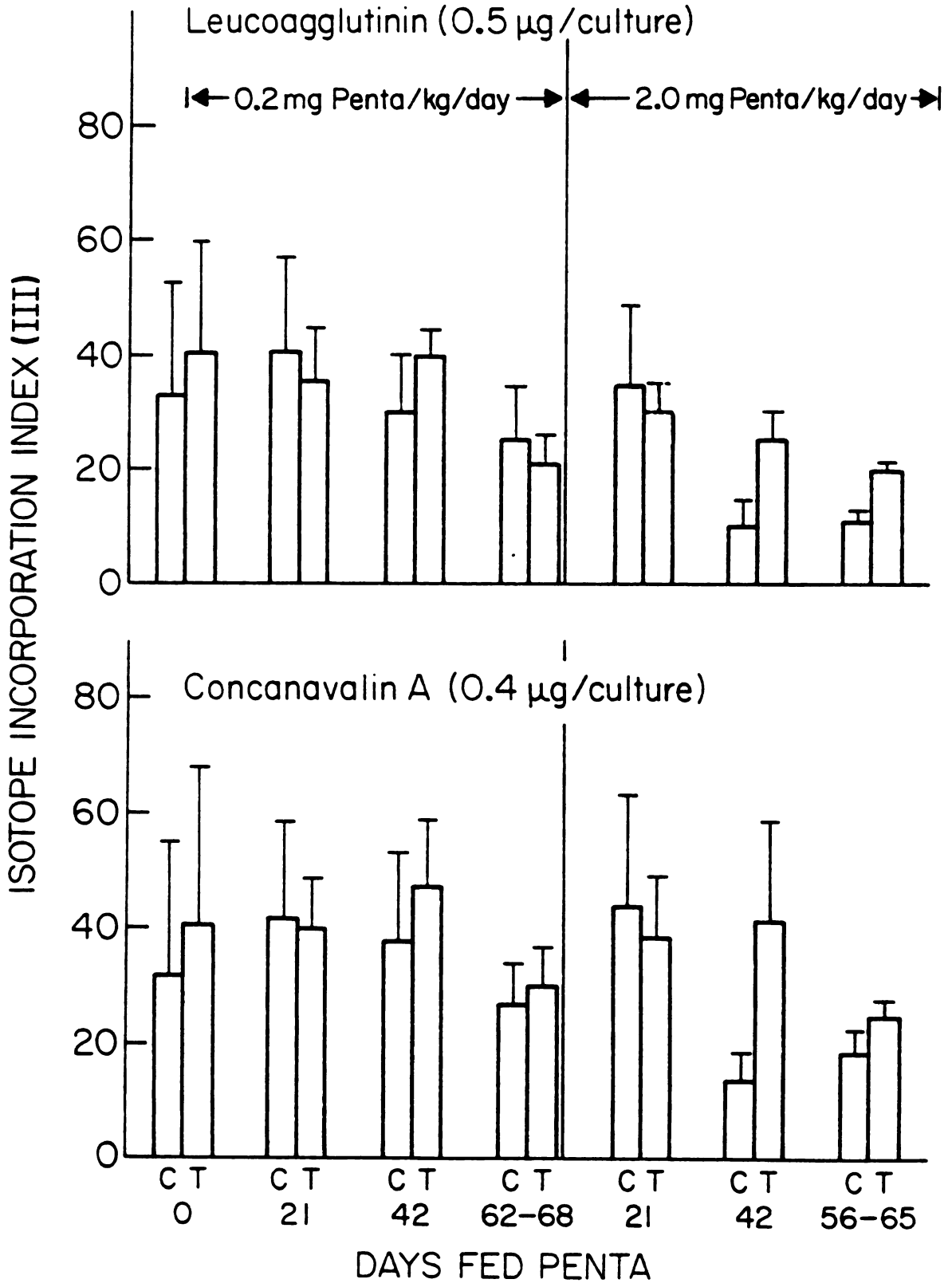

0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
LYMPHOCYTES x 10³/µl
0
1
2
3
4
5
6
7
8
Normal Range
sIg
En - RFC
C T
0
C T
14
C T
28
C T
42
C T
56
C T
63-84
C T
13
C T
26
C T
39
C T
52
C T
56-65
DAYS FED PENTA

Figure 1.5. Lymphoblastogenic Response to Optimal Concentrations of LA and Con A in Control (C) and Penta-treated (T) Cattle. A) Data Expressed as an Isotope Incorporation Index (III = Mean Counts/Minute in Mitogen Stimulated Cultures Divided by the Mean Counts/Minute in Control Cultures).

B) Data Expressed as Net Counts/Minute (NCPM) = Counts/Minute in Mitogen Stimulated Cultures Minus the Mean Counts/Minute in Control Cultures. Each Bar is the Mean + SEM of all Data Collected Between the $\bar{T}$imes Indicated.


A
Leucoagglutinin (0.5 µg/culture)
0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
Concanavalin A (0.4 µg/culture)
ISOTOPE INCORPORATION INDEX (III)
0
20
40
60
80
C T
0
21
42
62-68
21
42
56-65
DAYS FED PENTA

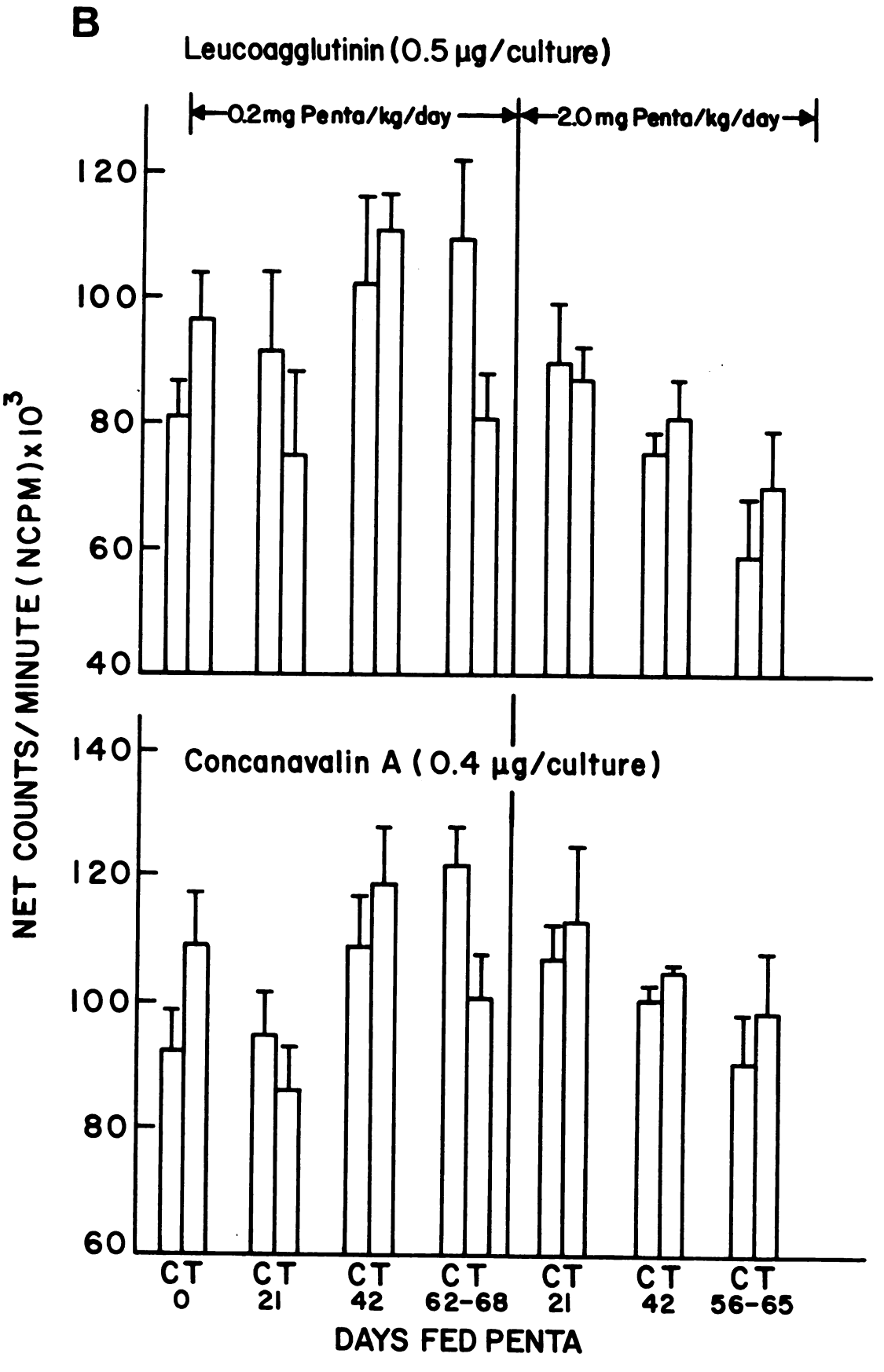

B
Leucoagglutinin (0.5 µg/culture)
0.2mg Penta/kg/day
2.0mg Penta/kg/day
120
100
80
60
40
NET COUNTS/MINUTE (NCPM) x 10³
Concanavalin A (0.4 µg/culture)
140
120
100
80
60
CT 0
CT 21
CT 42
CT 62-68
CT 21
CT 42
CT 56-65
DAYS FED PENTA

Table 1.1

In Vivo Cell-Mediated Immunity in Cattle Fed Technical Pentachlorophenol Subchronically[a]

Experimental Group	24 hour Response	48 hour Response
Control (3)[b]	42.9 ± 17.5	54.8 ± 28.8
Treated (4)	46.6 ± 14.2	48.4 ± 8.6

Values represent mean area (cm^2) ± SE of skin reaction at 24 and 48 hours following intradermal injection of PPD in BCG sensitized cattle.

a = 0.2 mg Penta fed/kg body weight/day for 60 days followed by 2.0 mg Penta fed/kg body weight/day for 75 days.

b = Number of cattle tested.

to BCG did not react to the PPD injection. The area of induration was comparable between control and penta-treated cattle. A histopathologic study of the PPD injection site revealed a marked infiltration of mononuclear cells in all animals.

Humoral immunity in control and penta-treated animals was tested by quantitating the serum immunoglobulins (IgG, IgM, IgA) and by evaluating antibody response following intravenous immunization with SRBC (Figure 1.6 and Table 1.2). The magnitude of the difference in serum IgG, IgM, and IgA, concentrations was similar in the two animal groups over time (Figure 1.6). Additionally, the mean peak anti-SRBC titer and the kinetics of the hemagglutinating antibody response were similar in both control and penta-fed cattle (Table 1.2).

Neutrophil Function Tests

Neutrophils isolated from peripheral blood were tested for their phagocytic and chemiluminescent responses following in vitro challenge with opsonized latex particles or opsonized zymosan. The percentage of phagocytizing neutrophils from control and penta-treated cattle are shown in Figure 1.7. Neutrophils from control cattle were incubated with latex particles opsonized with control sera, while neutrophils from penta-treated animals were challenged with latex particles opsonized in sera from penta-treated cattle. Additionally, neutrophils from penta-treated cattle were challenged with latex particles opsonized with control sera. The difference in the phagocytic response of neutrophils from control and penta-treated cattle was comparable over the duration of the dosing regime regardless

Figure 1.6. Serum Immunoglobulin Concentrations of IgG, IgM and IgA in Control and Penta-treated Cattle. Each Graphed Point Represents the Mean ± SEM of all Data Collected Between the Times Indicated.

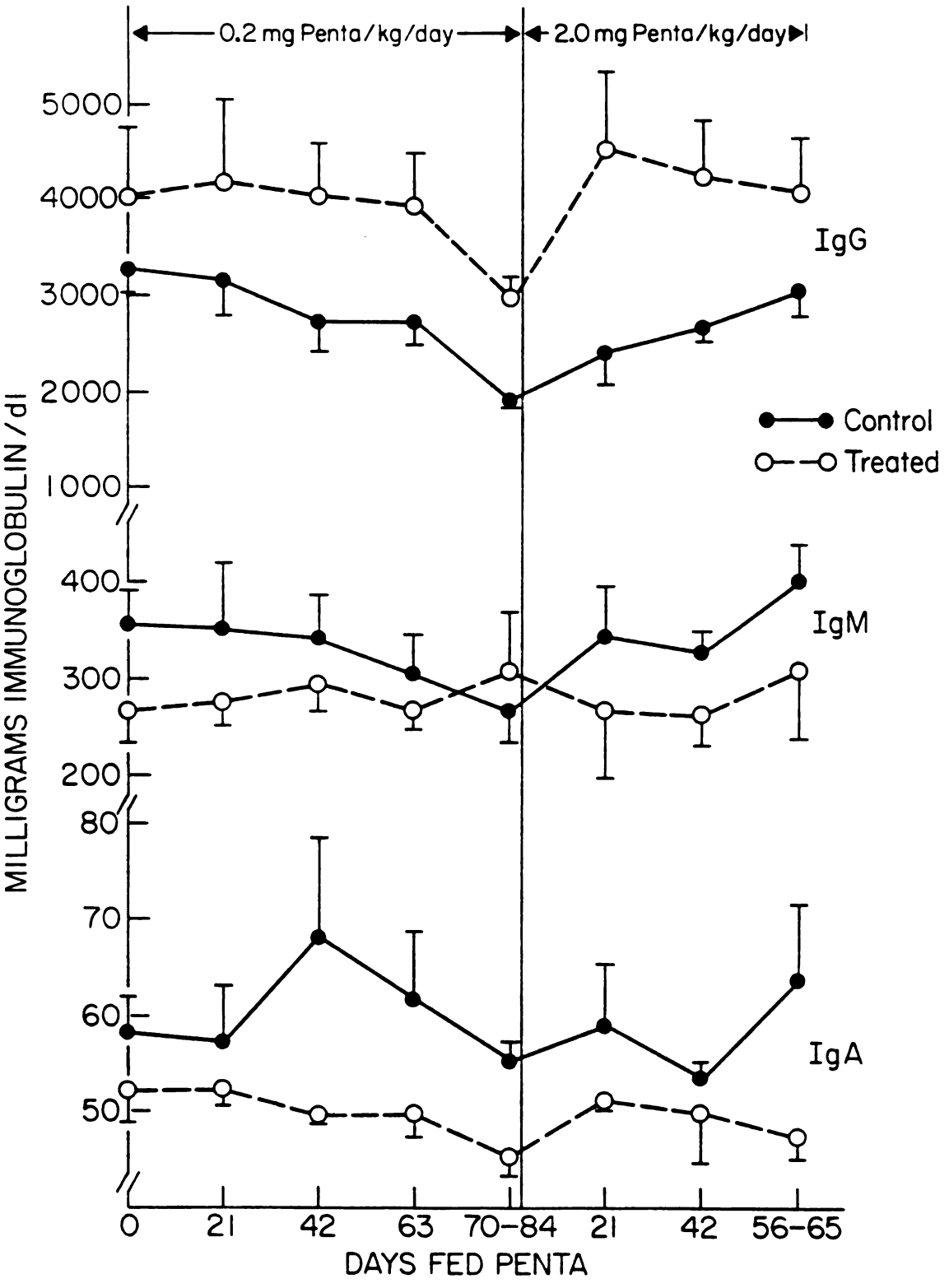

0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
5000
4000
3000
2000
1000
400
300
200
80
70
60
50
IgG
IgM
IgA
Control
Treated
MILLIGRAMS IMMUNOGLOBULIN / dl
0
21
42
63
70-84
21
42
56-65
DAYS FED PENTA

Table 1.2

Antibody Formation in Response to Injected Sheep Red Blood Cells[a] (SRBC) in Holstein Cattle Subchronically[b] Exposed to Commercial Pentachlorophenol.

SRBC	Control	Treated
Pre-immunization	2.75 ± 0.48[c]	2.25 ± 0.48
Day 4, post-immunization	4.00 ± 0.70	4.25 ± 0.63
Day 7, post-immunization	4.25 ± 0.86	4.50 ± 0.50
Day 10, post-immunization	4.00 ± 0.70	4.50 ± 0.29
Day 14, post-immunization	3.67 ± 0.90	4.67 ± 0.34
Day 35, post-immunization	3.50 ± 0.64	3.50 ± 0.50

a = 2 ml of a 10% v/v SRBC in saline injected intravenously.

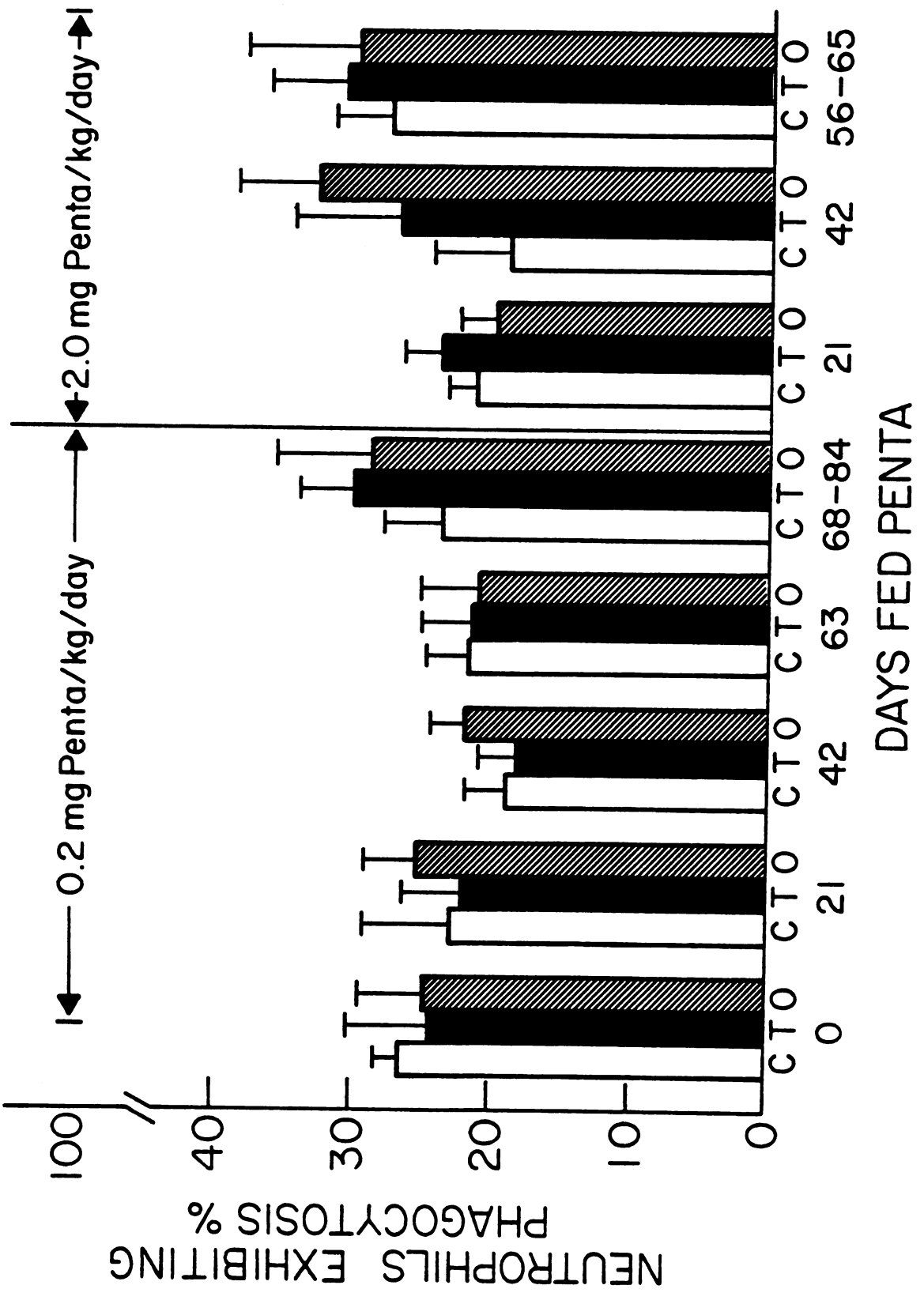
b = 0.2 mg Penta fed/kg body weight/day for 60 days followed by 2.0 mg Penta fed/kg body weight for 75 days.

c = Values represent mean titer ($\log_2$) ±SE for 4 cattle except day 14 figures which represents 3 cattle.

Figure 1.7. Neutrophil (PMN) Function Expressed as a Percent of Cells Phagocytizing Opsonized Latex Particles *In Vitro*.

C = Neutrophils from Control Cattle Opsonized with Serum from Control Cattle.
T = Neutrophils from Treated Cattle Opsonized with Serum from Treated Cattle.
0 = Neutrophils from Treated Cattle Opsonized with Serum from Control Cattle.

Each Bar Represents the Mean ± SEM of all Data Collected Between the Times Indicated.


NEUTROPHILS EXHIBITING
PHAGOCYTOSIS %
100
40
30
20
10
0
0.2 mg Penta/kg/day
2.0 mg Penta/kg/day
C T O C T O C T O C T O C T O C T O C T O C T O C T O C T O
0
21
42
63
68-84
21
42
56-65
DAYS FED PENTA

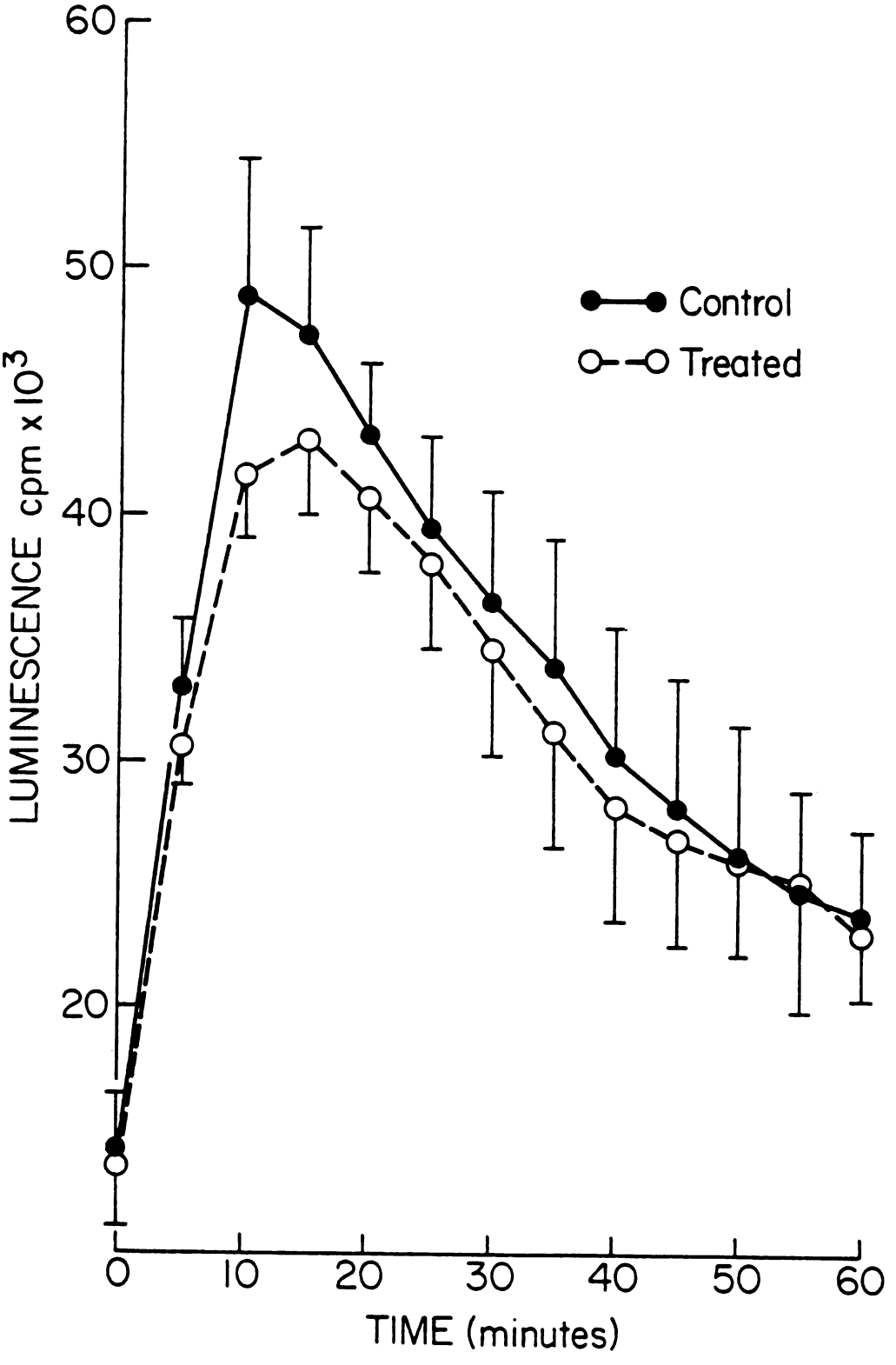
of the source of opsonizing serum. The results in Figure 1.8 depict the chemiluminescent response of neutrophils from cattle on the day of necropsy. Neutrophils from the penta-treated cattle exhibited a chemiluminescent response similar to neutrophils from control cattle, both in peak height and in the response kinetics.

Histology

Gross and histopathological evaluation of the spleen, thymus, and lymph nodes from control and penta-treated cattle revealed tissues which were normal in size, appearance, and histological architecture. Spleen weights of treated and non-treated cattle had identical ranges (900-1120 g) and means (1000 g). The thymus from penta-treated cattle had some peripheral lobules which were replaced by variable amounts of adipose tissue; however, similar findings were observed in control cattle.

During the course of the experiment some cattle in both the treatment and control groups periodically experienced an upper respiratory infection (URI) of unknown but consistent etiology, including elevated body temperature, duration of 1-2 days, and mild anorexia. The episodes of URI did not appear dose-related and responded to drug therapy.

Figure 1.8. *In Vitro* Light Emitting Profiles (Chemiluminescence) of Neutrophils (PMN) Phagocytizing Opsonized Zymosan A. This Assay Was Performed on the Last Day of Penta Exposure Using Cells from Both Control and Penta-treated Cattle. Each Point Represents the Mean ± SEM.


60
50
40
30
20
LUMINESCENCE cpm x 10³
Control
Treated
0
10
20
30
40
50
60
TIME (minutes)

DISCUSSION AND CONCLUSIONS

Immunodulation is the contemporary term ascribed to variation (either enhancement or suppression of the immune system) resulting from genetic, physiologic, or environmental influences. In this study, several immunologic paramaters were evaluated in lactating dairy cattle fed 0.2 mg technical pentachlorophenol per kg body wt/day for 75-84 days, followed by 2.0 mg/kg body wt/day for 56-60 days. The major physiologic compartment used in these investigations was blood which contained a total (acid hydrolyzed) PCP concentration at steady state of 2.9 ppm and 12.5 ppm during the 0.2 mg exposure period and 2.0 mg exposure period, respectively (Kinzell, 1981). Analysis of the bovine immune response included both quantitative and functional studies of lymphocytes and neutrophils, as well as serum-borne immunoreactive substances. No penta-induced cytotoxicity, alteration of lymphocyte surface markers, or changes in DNA or protein snythesis in lymphocytes were observed.

Although the immune competence values in penta-fed cattle were within normal limits, one of the treated cattle experienced clinical mastitis during the 0.2 mg/kg body wt/day exposure period. Laboratory studies during the infection demonstrated increases in several *in vivo* host defense parameters, including WBCs, percentage of PMNs, and serum IgG. Also, two treated cattle with an upper respiratory infection of unknown etiology responded

rapidly to antibiotic chemotherapy. Subsequent infections were not evident, further supporting the maintenance of immunologic competency in these animals. Interestingly, the mitogenic response of lymphocytes at the time of infection was reduced, but not to the extent to be considered abnormal. Several reports have described a reduced blastogenic response in experimental animals and in humans with concurrent infections (Oppenheim et al., 1975). Presumably, serum factors manifested during infectious events are contributory to the reduced lymphoblastogenic response, since incubation of normal lymphocytes with serum from infected animals can markedly reduce the lymphoblastogenic response. In these cattle, the LA and Con A induced lymphocyte blastogenic response increased when the infection cleared following antibiotic therapy.

Histologic examination of lymphoid tissues revealed no lesions; however, it cannot be conclusively stated that the functional status of these specific lymphoid tissues was intact. Considerable evidence indicates that the chlorophenols (i.e. pentachlorophenol and tetrachlorophenol) do not accumulate in bovine tissues whereas the polychlorinated dioxins and furans steadily accumulate primarily in the liver and body fat during continuous exposure (Firestone et al., 1979). In these cattle, PCP levels in the thymus and spleen at terminus average 2.8 and 3.4 ppm, respectively (Kinzell, 1981). Analysis of the total dioxin (sum of octa-, hepta-, and hexa-dioxin) concentration in the livers of the four penta-fed cattle was 145 ppb. Dioxin concentrations in various lymphoid tissues were not determined.

Whereas these data shed some light on the disposition of these chemicals, they are not adequate for establishing the level of exposure of different immunologic reactive cells in these tissues. Such studies (see Chapter 2) provide insight into the time required for any one of these chemicals to reach a toxic threshhold concentration in the non-blood immune compartments.

While these studies were in progress, McConnell et al. (1980) reported that in 10-14 month-old Holstein heifers fed for 160 days with approximately 10 times more penta than cattle in this experimental regime received, exhibited some hematologic changes. A limited immunologic evaluation in these cattle also revealed a dose-related (attributed to technical pentachlorophenol) enhancement of mitogen-induced lymphoblastogenesis and a decrease in absolute and relative thymus weight[1]. However, no additional alterations in immune competence as measured by the SRBC hemagglutinin response, serum complement (C3) level, mixed lymphocyte culture response, immunoglobulin levels and presence of ANA were noted.

It is interesting to note that at the lowest level of technical pentachlorophenol used in the McConnell et al. (1980) study (a mixture of technical and analytical pentachlorophenol in a ratio of 10:90), approximately the same total amount of technical pentachlorophenol was administered per animal (84 grams) as in this study (68 grams). Although an additional

[1]Comparatively, it was not possible to record accurate thymus weights because of the difficulty in excising the whole gland from older cattle.

13.5 to 18 mg/kg body weight of analytical pentachlorophenol was also contained in the McConnell dosing regime per day, no immunomodulation or effect on hematology was seen above that which was observed in cattle which were dosed with 100% analytical pentachlorophenol alone.

Immunotoxicologic studies are rapidly emerging as an approach in which various chemicals in toxicologic studies are being evaluated. At present, knowledge regarding mechanisms of host defense, immunoregulatory events, and immune effector mechanisms in humans and rodents far exceeds knowledge of these same processes in other species, including food producing animals such as cattle. Other immunotoxicological studies involving cattle include studies by Kateley and Bazzell (1978) who investigated cattle subchronically exposed to polybrominated biphenyls (PBBs), and McConnell et al. (1980) who have investigated the pathophysiologic effects of both analytical and technical pentachlorophenol in young cattle. In addition, Haggard et al. (1980) recently reported that the feeding of excessive iodine induced immunomodulation. However, many more chemicals have been identified in cattle tissues resulting from environmental exposure to pesticides, heavy metals, and solvents (EPA 1977) which need to be evaluated for immunotoxicity. Unfortunately, establishing immunotoxicity is a difficult process primarily because of the multifaceted nature of the immune system. There currently is a lack of agreement as to the importance, relevance and interpretation of results derived from individual testing procedures. However, when individual in vitro and in vivo immunoassays are combined and

used as a profile, the interpretation of the data generally permits a more thorough evaluation of immune status. The present investigation evaluated several aspects of immunity in lactating cattle exposed subchronically to penta. Since there was no evidence for either a quantitative or functional immunologic defect in cattle with blood levels of 12.5 ppm PCP, it can be concluded that there is no penta-induced immune deficiency in cattle with this body burden.

Chapter 2

TECHNICAL AND ANALYTICAL PENTACHLOROPHENOL IN DAIRY CALVES: A CLINICOPATHOLOGICAL AND IMMUNOTOXICOLOGICAL EVALUATION

INTRODUCTION

Pentachlorophenol is a commonly used wood preservative with widespread uses on modern dairy farms (Shull et al., 1981). Because of the high potential for exposure of confined cattle to the chemicals with are present in commercial preparations of pentachlorophenol, several experimental investigations have been conducted to ascertain possible toxic effects (Firestone et al., 1979; McConnell et al., 1980; Kinzell et al., 1981; Hughes et al., 1982). Whether or not pentachlorophenol is toxic to the various bovine host defense mechanisms has been considered in some of this research. McConnell et al. (1980) showed that contaminants in technical grade pentachlorophenol (tPCP) fed at high levels are immunomodulating in yearling cattle. These contaminants include several chlorodibenzo-p-dioxin (CDD) and chlorodibenzofuran (CDF) congeners and hexachlorobenzene (Firestone et al., 1972; Firestone et al., 1979; Kinzell et al., 1981). The well-known 2, 3, 7, 8-tetra CDD which is not present in tPCP preparations (Kinzell et al., 1981) is a known immunosuppressant (Gupta et al., 1973). Hexachlorobenzene has also been shown to be immunosuppressive (Loose et al., 1977).

In a previous study in which lactating dairy cattle were fed for five months a level of tPCP intended to typify actual farm exposure (Kinzell et al., 1981), no apparent compromise of the immune system was observed (Chapter 1). To date, there is no information on the immunotoxicity of PCP or its contaminants in calves. Thomas et al., (1977) reported

high mortality rates of calves on a few farms where there appeared to be an overuse of chemically treated wood. For several reasons, calves could be more sensitive to these chemicals than their adult counterparts. First, young animals are generally more sensitive than adults to many toxicants. For example, piglets have been shown to be more sensitive to PCP than adult swine (Schipper, 1961; Ryan, 1983). Second, the immune system in calves is not entirely developed at birth (Wells et al., 1977; Jensen, 1978; Renshaw et al., 1978; Tizard, 1982) which could exacerbate the immunotoxic potential of toxicants such as those in tPCP. Third, the metabolic fate of PCP or the contaminants could be quite different in monogastric calves versus ruminant adults due to pre-absorption metabolism in the latter. It is well-known that the microflora of the GI tract, particularly the rumen, significantly affects the fate and toxicity of many xenobiotics (Williams, 1977).

The results of immunologic and clinical testing are given in this chapter. Additional data collected from the calves in this study are reported elsewhere (Hughes, 1982).

MATERIALS AND METHODS

Technical grade pentachlorophenol (tPCP)[6] was generously supplied by the American Wood Preservers Institute and represented a commercial composite from the three major manufacturers of pentachlorophenol. Analytical grade pentachlorophenol (aPCP)[7] was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

The chlorophenol and chlorodibenzodioxin (CDD) composition of the tPCP was previously reported (Kinzell et al., 1981). The aPCP contained 99% pentachlorophenol and 0.98% tetrachlorophenol. The CDD concentrations (ppm) were: octa-CDD, 1.2; hepta-CDDs, 1.8; and hexa-CDDs, 0.2 (R. Johnson, Dow Chemical Company, personal communication).

Other chemicals and reagents included: Ficoll, and leukoagglutinin (LA); (Pharmacia Fine Chemicals, Piscataway, NJ), Hanks balanced salt solution (HBSS), Ca^{2+} - and Mg^{2+} -free HBSS, sheep red blood cells (SRBs), fetal calf serum, and RPMI-1640 with 25 mM Hepes (Microbiological Associates, Walkerville, MD), Vibrio cholerae neuraminidase (VCN), penicillin and streptomycin (Gibco,Grand Island, NY), fluroescein labeled (FITC) rabbit and antibovine-immunoglobulin (Ig) reagents and single radial immunodiffusion agarose plates for serum Ig concentrations (Miles Laboratories Inc., Elkhart, IN), [^{3}H] thymidine ([^{3}H] TdR), (New England Nuclear Corp., Boston, MS), Hypague (Sterling Organics, New York), and zymosan A (Sigma

[6]Lot no. MB-528, Vulcan Chemical Co., Birmingham, AL.
[7]Lot No. 032487

Chemical Co., St. Louis, MO).

Treatment of Calves

Fifteen Holstein-Friesian bull calves, ranging from three to eleven days of age were randomly assigned to the following treatment groups: control, 1.0 mg aPCP/kg body weight, 1.0 mg tPCP/kg, 10.0 mg aPCP/kg, and 10.0 mg tPCP/kg per day. These levels of exposure were maintained for 43 days with the exception of the first five days when the calves received 2.0 and 20.0 mg PCP/kg. The level of exposure was cut back when two calves died, one each in the aPCP and tPCP groups as a result of acute pentachlorophenol toxicity. Two replacement calves were obtained, however the replacement calf in the aPCP group also succumbed.

Pentachlorophenol, either analytical or technical grade, was dissolved in corn oil. The pentachlorophenol in corn oil was then added to milk so that the calves were fed an amount of milk equivalent to 8% of each animal's body weight every day. The calves were dosed twice daily. Since tPCP is 85% PCP and aPCP is 99% PCP, the dose of PCP was adjusted so that equivalent amounts of PCP per kg body weight was fed.

The calves were weighed every five days and the dose adjusted accordingly. The calves were individually housed in pens in a warm enclosed barn. Water was provided at all times and starting at day 20, the calves were fed a calf starter ration ad libitum.

Sample Specimens

Blood for hematologic, clinical chemistry and immunologic measurements was collected from each calf during the last week of the trial. Complete blood counts were performed with EDTA-anticoagulated blood, clinical chemistry with serum and immunologic studies with serum or cells obtained from heparinized blood. Urine samples were obtained by subsampling urine collected over a 24-hour period while the calves were in metabolism cages.

Hematology and Clinical Chemistry

Hematologic values including red blood cell (RBC) count, hemoglobin (HGB) content, and white blood cell (WBC) count were determined with a Coulter counter (model S). Packed cell volumes (PCV) were determined by micro-hematocrit centrifugation. White blood cell differential analyses and platelet estimations were derived from Wright-stained blood smears.

All clinical chemistry analyses with the exception of sorbitol dehydrogenase, gamma-glutamyl transferase (GGT) and cortisol were performed on a Computerized Sequential Multiple Analyser (SMAC, Technicon). Sorbital dehydrogenase and GGT were analysed using a Gemsaec centrifugal analyzer (Electro-Nucleonics, Inc). Cortisol levels were determined by radio-immunoassay[8].

Leucocyte Preparations

Lymphocytes and neutrophils were separated from heparinized blood on Ficoll/Hypague (F/H) cushions (Boyum, 1968),

[8]Michigan State University Veterinary Diagnostic Lab

and processed according to the method described previously (see Methods, Chapter 1) with one change. Remaining erythrocytes were removed by hypotonic shock utilizing sterile double distilled water instead of 0.85% NH_4Cl.

Membrane Receptor Assays

Three membrane receptors were assayed on lymphocytes obtained from the circulating blood and from spleen, thymus and lymph node tissue. Sheep erythrocyte receptors or erythrocyte (E) binding was determined by a rosette assay using sheep red blood cells (SRBC) treated with (VCN) according to the procedure described previously (Chapter 1).

Complement (C) bearing lymphocytes were detected by rosetting procedures with antigen-antibody-C complexes. The complexes consisted of human type O erythrocytes sensitized with a subagglutinating dilution of rabbit anti-human RBC antibody and a subhemolytic concentration of mouse complement (EAC) (Niblack and Gengozian, 1976) surrounding lymphocytes with complement receptors.

Surface immunoglobulins (Ig) were enumerated with a polyspecific fluorescein-labeled (FITC) rabbit antibovine Ig reagent according to the procedure described previously (Chapter 1).

Lymphocyte and Neutrophil Function Tests

Mitogen-induced blastogenesis of lymphocytes as measured by $[^3H]$ thymidine uptake, was performed in triplicate by a microculture procedure (Douglas, 1971). One mitogen, leukoagglutinin (LA), was used to stimulate the lymphocytes at one optimal

concentration of LA (0.5 ug) and two suboptimal LA concentrations. Otherwise, the procedure was as described previously (Chapter 1).

Serum IgG, IgM, and IgA were determined by radial immunodiffusion (Mancini et al.,1965; Butler, 1971) with commercial reagents. Each Ig was quantified three different times for each calf over the last ten days of the trial.

During the last 30 days of PCP exposure, the primary and anamestic (secondary) response to human red blood cells (HRBC) was evaluated.Three ml of 10% (V/V) HRBC in saline was injected IV followed by 1.5 ml of 10% (V/V) HRBC injected 15 days later. Serum was collected before inoculation as well as on day 4, 9, 14, 19, 24, and 29 after the first inoculation. HRBC agglutinin titers were determined with heat-inactivated (56^{o}C for 30 min) serum. The highest serum dilution exhibiting macroscopic agglutination was recorded as the titer.

Neutrophil chemiluminescence studies (Allen et al., 1972); Rosen and Klebanoff, 1976; Andersen et al., 1977) were performed on the day of necropsy. In the assay, 2 ml of polymorphonuclear neutrophils (PMN) containing 1 x 10^{6} cells per ml were added under red light to dark-adapted scintillation vials containing 0.5 ml bovine serum opsonized zymosan A (Saccharomyces cerevisiae). PMN luminescence was measured over a one hour period in a Searle Isocap 300 liquid scintillation counter adjusted to detect visible light (noncoincident mode).

Histology

At necropsy, liver, kidney, spleen, thymus and brain

were removed intact, trimmed of excess fat and connective tissue, and weighed. Bone marrow and a representative mesenteric lymph node were also taken for histopathologic examination. All tissues were fixed in 10% buffered formalin. Paraffin embedded sections of these tissues were stained with hematoxylin and eosin before histologic examination. A gross and histopathologic examination were performed on each animal. These results along with neonatal toxicity and thyrotoxicity data, as well as PCP residue analysis are reported by Hughes, (1982).

Statistical Analysis

Data were analyzed by regression analysis and analysis of variances with Genstat V system at Michigan State University, East Lansing, Michigan. For some analyses, Dunnett's test was used (Gill, 1978).

RESULTS

Hematology and Clinical Chemistry

Venous blood and urine obtained during the final week of PCP exposure were used for various hematologic and clinical chemistry measurements (Tables 2.1, 2.2 and 2.3). All of the hematologic values were either within the normal range of values for calves cited by Schalm et al., (1975) or did not differ significantly from controls.

Pentachlorophenol treatment decreased serum concentrations of total protein and albumin (Table 2.2) and increased the activity of gamma-glutamyl transferase (GGT) (Table 2.3). Compared to control values for all of these parameters, the greatest differences were found in the calves fed 10 mg tPCP/kg. No other blood or urine parameter differed significantly from controls. Whereas the decreases in both serum total protein and albumin were dose-dependent ($P < .10$), only the protein values were below the normal range for bovines as reported by Duncan and Prasse (1977). Although there appeared to be a trend of decreasing globulin concentrations with increasing PCP exposure, the differences were not statistically significant. The increased activity of serum GGT was dose-dependent ($P < .10$) and reached a level in the 10 mg/kg tPCP group that exceeded the normal range in cattle by 10 times. (Michigan State University Clinical Laboratory, personal communication).

Membrane Receptors

The percent and number of surface receptors present on

Table 2.1. Results of Hematologic Examination of Blood From Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Measurement	Units	Control x ± SE		Low Analy. x ± SE		Low Tech. x ± SE		High Analy.[b] x ± SE		High Tech. x ± SE	
White blood cells (WBC)	n/ul	9,583	636	6,933	650	10,583	1475	6,250	550	9,850	1765
Lymphocytes	n/ul	4,058	1210	4,424	361	5,271	1185	3,012	18	4,666	1423
Polymorphonuclear neutrophils (PMN)[c]	n/ul	4,580	1145	1,852	670	4,608	169	3,128	540	4,687	765
Non-segmented neutrophils	n/ul	35	18	20	11	0	0	16	16	312	312
Monocytes[c]	n/ul	769	234	409	138	606	138	94	45	186	78
Eosinophils	n/ul	106	82	217	102	32	32	0	0	0	0
Basophils	n/ul	36	35	11	11	66	39	0	0	0	0
Red blood cells (RBC)	nx10^6/ul	8.38	0.30	7.52	0.54	7.98	0.77	6.38	1.44	7.97	0.61
Hemoglobin (Hb)	g/dl	10.1	0.5	8.7	0.8	9.3	1.0	7.6	2.0	9.5	0.7
Packed cell volume (PCV)	%	29.9	1.9	25.7	1.4	27.4	2.8	23.0	6.9	28.5	2.7
Mean corpuscular vol.(MCV)	fl	30.5	0.8	29.3	1.2	29.0	1.0	30.8	2.2	31.3	0.3
Mean corpuscular Hb (MCH)	pg	12.2	0.3	11.6	0.3	11.6	0.1	11.9	0.4	12.0	0.1
Mean corpuscular Hb conc. (MCHC)	g/dl	40.7	1.3	40.2	0.7	41.3	1.6	39.3	1.5	39.4	0.3
Platelets[d]	est.	sl ↑		Ad.		Ad.		Ad.		Ad.	

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose for 43 days.

[b] n = 2; for all other groups, n = 3.

[c] Significant dose response ($P < 0.10$).

[d] Estimate (est) of platelet number, either adequate (Ad) or slightly increased (sl ↑).

Table 2.2. Results of Clinical Chemistry Analysis of Serum From Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Measurements	Units	Control[b] $\bar{x} \pm SE$	Low Analy. $\bar{x} \pm SE$	Low Tech. $\bar{x} \pm SE$	High Analy. $\bar{x} \pm SE$	High Tech. $\bar{x} \pm SE$
Blood urea nitrogen (BUN)	mg/dl	9.0 1.5	9.0 2.3	7.7 0.9	15.0 3.2	9.0 1.2
Glucose	mg/dl	92 12	107 13	110 21	105 25	111 58
Total Protein[c]	g/dl	6.0 0.1	5.3 0.1	5.3 0.2	5.3 0.3	4.6 0.4[e]
Albumin[d]	g/dl	3.3 0.1	3.3 0.03	3.1 0.1	3.2 0.2	2.7 0.1[f]
Globulin	g/dl	2.7 0.1	2.1 0.1	2.2 0.1	2.1 0.2	1.9 0.4
Calcium	mg/dl	10.0 0.2	10.4 0.4	10.2 0.2	10.2 0.3	9.2 0.6
Phosphorus	mg/dl	6.1 0.3	6.3 0.4	6.9 0.6	6.8 0.6	5.8 0.7
Sodium	mEq/L	141 1.5	142 0.9	140 1.0	142 2.0	139 2.2
Potassium	mEq/L	4.4 0.2	4.9 0.2	4.8 0.1	4.8 0.2	4.9 0.4
Chloride	mEq/L	101 0.9	104 1.3	103 0.6	101 1.8	102 4.3
Carbon Dioxide	mM/L	29 1.0	29 1.0	28 0.6	28 1.5	27 3.9
Electrolyte Gap[g]	none	11.3 1.2	9.7 0.7	9.0 1.0	13.0 2.9	9.7 1.8
Uric Acid	mg/dl	0.8 0.2	1.0 0.1	0.9 0.2	1.6 0.7	1.0 0.3

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose, for 43 days.

[b] n = 3.

[c] Significant dose response ($P < 0.10$).

[d] Significant dose response ($P < 0.01$).

[e] Significantly different from control ($P < 0.05$).

[f] Significantly different from control ($P < 0.01$).

[g] Is the difference between the unmeasured anions and cations in the serum.

Table 2.3. Results of Clinical Chemistry Analysis of Serum and Urine From Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Measurement	Units	Control $\bar{x} \pm SE$	Low Analy. $\bar{x} \pm SE$	Low Tech. $\bar{x} \pm SE$	High Analy. $\bar{x} \pm SE$	High Tech. $\bar{x} \pm SE$
Creatinine	mg/dl	0.8 0.1	1.0 0.1	0.8 0.03	1.2 0.4[b]	0.6 0.0
Cortisol	ng/ml	2.7 0.3	ND	ND	4.0 1.0[b]	3.3 0.9
Sorbitol dehydrogenase(SDH)	IU/L	17 4	15 3	23 4	31 21	61 20
Gamma-glutamyl transferase (GGT)[c]	IU/L	18 2	17 4	29 4	30 7[b]	93 25[d]
Aspartate aminotransferase (AST)	U/L	44 5	48 1	47 4	30 13	94 37
Alanine aminotransferase (ALT)	U/L	24 1	17 4	11 5	23 3	23 5
Lactate dehydrogenase (LH)	U/L	818 33	766 12	895 26	947 305	1438 386
Alkaline phosphatase (ALP)	U/L	226 63	224 55	231 35	194 77	115 18
Creatine kinase (CK)	U/L	77 15	187 88	108 7	41 21	604 531
Total bilirubin	mg/dl	0.2 0.03	0.1 0.03	0.1 0.03	0.1 0.0	0.1 0.03
Triglyceride	mg/dl	37 8	38 10	41 17	21 9	12 1
Urine protein	mg/24 hrs	430 22	570 238	248 22	309 119[b]	479 85[b]
Urine specific gravity	none	1.013 0.005	1.012 .002	1.013 0.001	1.013 0.002[b]	1.012 0.0005[b]
Urine creatinine	mg/24 hrs	1676 135	1739 188	1461 168	1280 399[b]	1100 99[b]
Creatinine clear.(24 hr)ml blood cleared/min[e]		141 15	125 12	134 20	104 2[b]	127 12[b]

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose, for 43 days.

[b] n = 2, for all other groups, n = 3.

[c] Significant dose response ($P < 0.01$).

[d] Significantly different from control ($P < 0.01$).

[e] Formula = $\frac{\text{(urine creatinine mg/dl) (urine flow ml/min)}}{\text{(serum creatinine mg/dl)}}$

ND = Not determined.

lymphocytes obtained from the blood, spleen, thymus and lymph node tissue of calves in the various treatment groups are presented in Tables 2.4 and 2.5. The numbers of En rosette-forming cells, EAC complement-bearing cells and surface Ig-bearing lymphocytes were comparable in all groups for all tissues.

Lymphocyte and Neutrophil Function Tests

One in vivo and three in vitro immunoassays were conducted to evaluate lymphocyte and neutrophil functions (Figures 2.1, 2.2 and 2.3, and Table 2.6). The blastogenic response of lymphocytes, which is an in vitro indicator of cell-mediated immunity, was measured in three lymphoid tissues (blood, spleen, and lymph node) from control and PCP-treated calves. The response was determined at three concentrations with the T cell dependent mitogen leukoagglutinin. Isotope incorporation ($[^3H]$ TdR) by cultured lymphocytes indicated that DNA synthesis in both aPCP and tPCP-treated calves was neither impaired or enhanced relative to controls (Table 2.6). Also, no differences were detected at the two submitogenic levels used (data not shown). Because insufficient thymus tissue was recovered from the calves in the 10 mg/kg tPCP group due to atrophy, no statistical analyses could be applied to the lymphoblastogenesis data obtained for the thymus. However, it appears that the response was drastically decreased by increasing PCP dose.

Humoral immunity was evaluated by quantitating serum levels of immunoglobulins G, M, and A and by evaluating the

Table 2.4. Peripheral Blood Lymphocyte Surface Markers in Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Experimental Group	En[b]		EAC[c]		sIg[d]	
	Number[e]	%[f]	Number	%	Number	%
Control	496 ±261	16.5 ± 8.0	266 ±123	5.9 ±1.1	657 ±162	17.5 ± 3.9
Low Analytical	444 ±117	11.6 ± 3.3	327 ±114	7.8 ±2.9	962 ±214	21.3 ± 3.0
Low Technical	492 ±178	8.8 ± 1.1	217 ±109	3.7 ±1.0	1359 ±666	23.0 ± 6.4
High Analytical[g]	324 ±285	10.7 ± 9.4	116 ± 26	3.8 ±0.8	652 ±263	21.6 ± 8.6
High Technical	650 ±323	12.6 ± 2.5	277 ±233	4.1 ±3.0	517 ±152	11.2 ± 0.4

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose for 43 days.

[b] En-rosettes which form with some lymphocytes and neuraminidase-treated SRBCs.

[c] EAC-rosettes which can be made to form with complement-bearing cells.

[d] sIg-lymphocytes which have surface immunoglobulins.

[e] Number of lymphocytes having each surface marker/ul whole blood ±SE.

[f] Percent of lymphocytes having each surface marker ±SE.

[g] n = 2; for all other groups, n = 3.

No significant differences found ($P > 0.05$).

Table 2.5. Lymphocyte Surface Markers in Lymphoid Tissues Obtained from Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Experimental Group	Lymph Node			Spleen			Thymus		
	En[b]	EAC[c]	sIg[d]	En	EAC	sIg	En	EAC	sIg
Control	28.4 ± 3.4	27.2 ± 2.0	17.2 ± 2.4	39.0 ±10.0	22.3 ± 8.7	17.5 ± 5.6	30.3 ± 4.9	0	<1.2
Low Analytical	37.6 ±12.7	25.8 ± 3.1	34.8 ± 3.0	31.5 ±12.3	19.8 ± 6.2	22.1 ± 5.1	36.8 ± 7.8	0	<1.0
Low Technical	36.5 ±12.3	18.2 ± 4.3	24.6 ± 5.8	39.3 ±11.4	13.9 ± 2.4	32.2 ± 5.5	37.3 ± 5.5	0	<1.0
High Analytical[e]	44.6 ±12.3	14.6 ± 7.4	30.8 ±10.4	23.7 ± 0.3	20.9 ± 7.9	27.8 ± 5.8	31.6 ± 7.6	0	<0.5
High Technical	24.5 ± 1.6	10.4 ± 2.0	15.8[f]	23.3 ± 3.4	13.8 ± 3.3	22.2 ± 3.4	27.6 ±12.1	1.4 ±1.2	<0.5

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose, for 43 days.

[b] Percent of viable lymphocytes which form rosettes with neuraminidase-treated SRBCs ±SE.

[c] Percent of viable lymphocytes which can be made to form erythrocyte amboceptor complement (EAC) rosettes with human RBCs ±SE.

[d] Percent of lymphocytes with surface immunoglobulins (sIg) ±SE.

[e] n = 2; for all other groups, n = 3.

[f] n = 1.

No significant differences found ($P > 0.05$).

Figure 2.1. Net Change in Serum Concentrations of IgG, IgM, and IgA Over a Ten Day Period in Calves Fed Analytical or Technical Grade Pentachlorophenol.

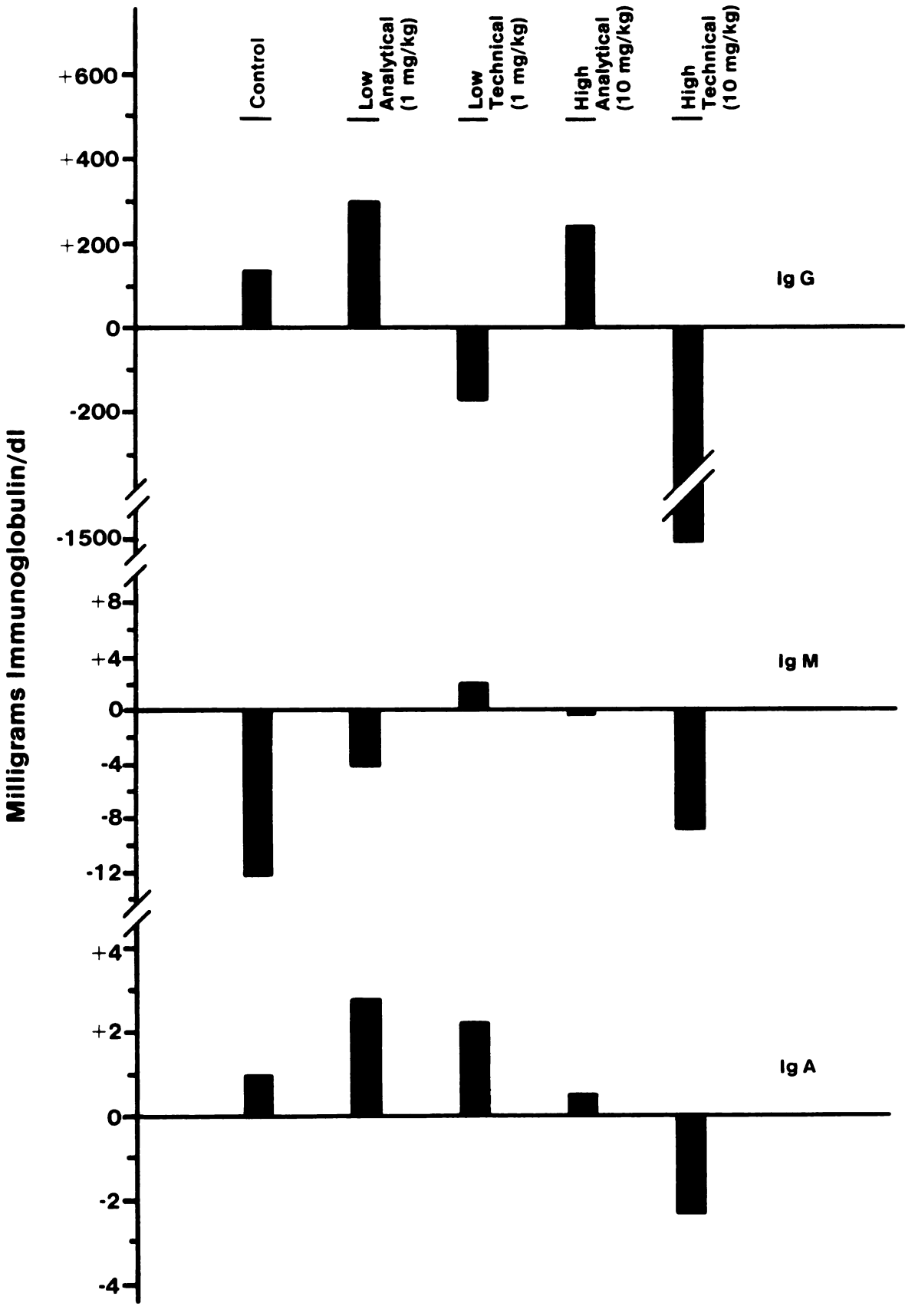

Control
Low Analytical (1 mg/kg)
Low Technical (1 mg/kg)
High Analytical (10 mg/kg)
High Technical (10 mg/kg)
Milligrams Immunoglobulin/dl
+600
+400
+200
0
-200
-1500
Ig G
+8
+4
0
-4
-8
-12
Ig M
+4
+2
0
-2
-4
Ig A

Figure 2.2. Primary and Secondary Antibody Response to Injected Human Red Blood Cells in Calves Fed Analytical or Technical Grade Pentachlorophenol.

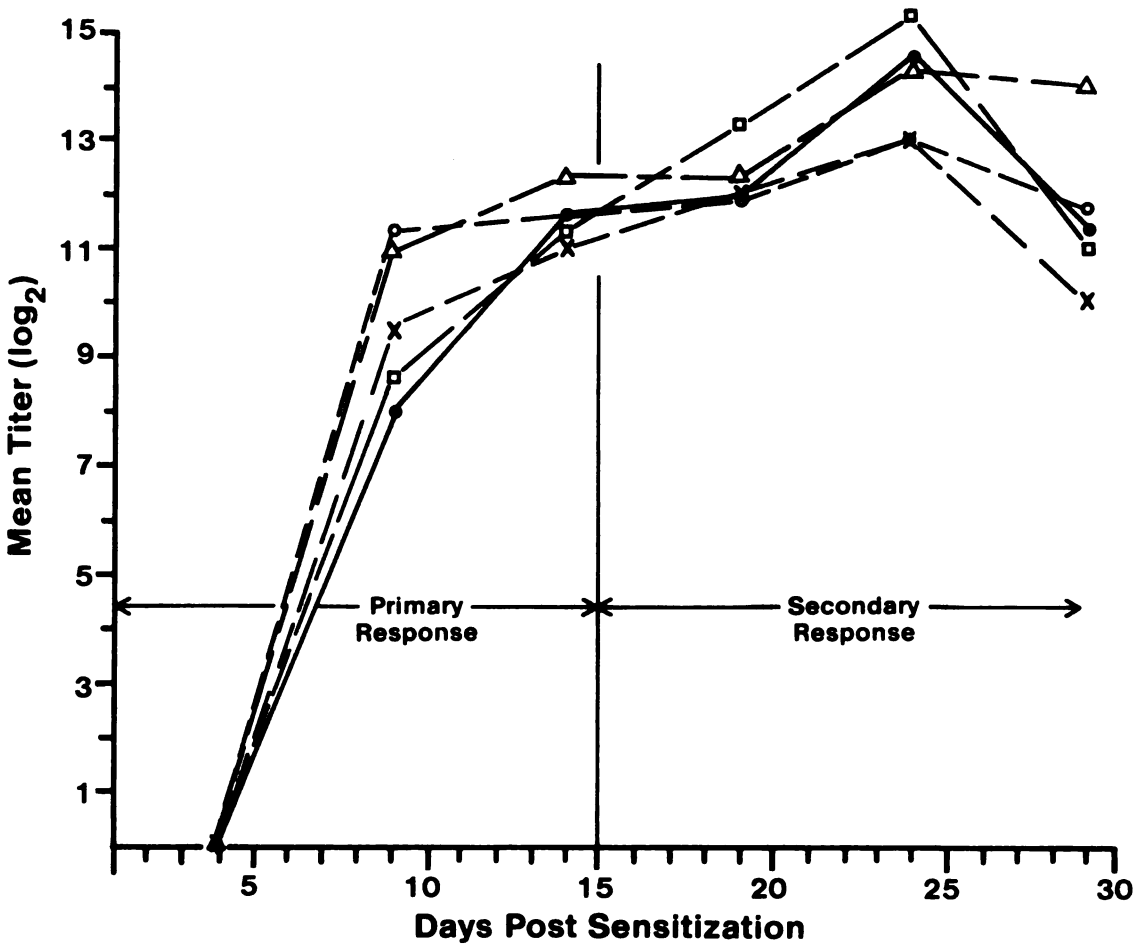

15
13
11
9
7
5
3
1
Mean Titer (log_2)
Primary Response
Secondary Response
5
10
15
20
25
30
Days Post Sensitization
Control
Low dose analytical 1 mg/kg body wt/day
Low dose technical 1 mg/kg body wt/day
High dose analytical 10 mg/kg body wt/day
High dose technical 10 mg/kg body wt/day

Figure 2.3. *In Vitro* Light-emission Profiles (Chemiluminescence) of Neutrophils (PMN) Phagocytizing Opsonized Zymosan A. The Plots Show the Mean Response for Each Treatment Group over an Hour Period.

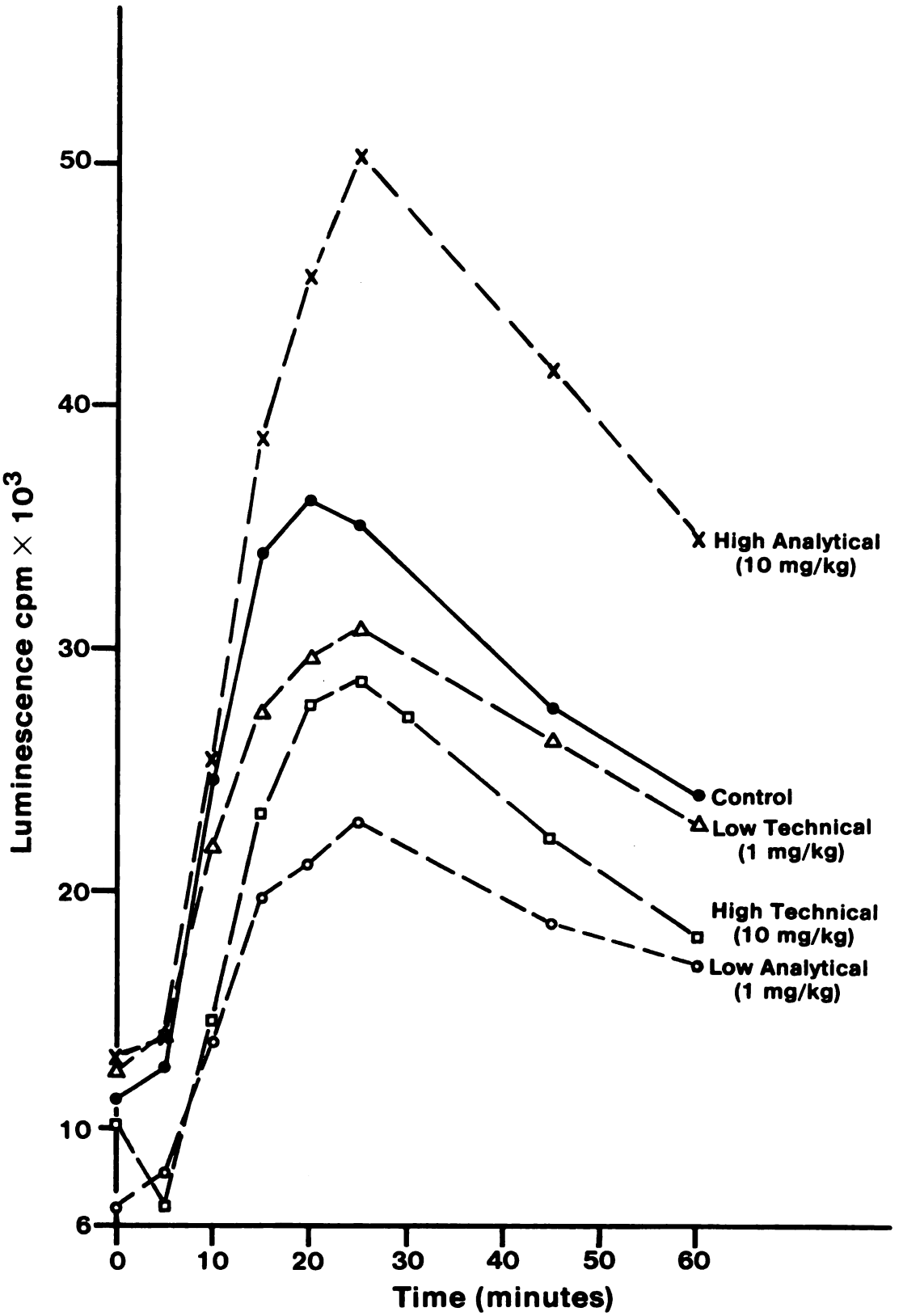

Luminescence cpm × 10³
50
40
30
20
10
6
0
10
20
30
40
50
60
Time (minutes)
High Analytical (10 mg/kg)
Control
Low Technical (1 mg/kg)
High Technical (10 mg/kg)
Low Analytical (1 mg/kg)

Table 2.6. Lymphoblastogenic Response To an Optimal Concentration of Leukoagglutinin (0.5 ug) in Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Experimental Group	Blood		Lymph Node		Spleen		Thymus	
	NCPM[b]	III[c]	NCPM	III	NCPM	III	NCPM	III
Control	47,565 ±11,244	8.77 ± 3.90	48,292 ±16.991	25.73 ±15.41	10,355	2.81[d]	12,726 ±12,158	2.65 ±1.42
Low Analytical	56,626 ± 5,150	11.83 ± 6.05	24,173 ± 3,329	19.28 ± 8.37	17,734 ± 743	6.27 ±3.61	N.D.	N.D.
Low Technical	51,312 ±17,585	13.44 ± 1.47	37,672 ±18,083	14.28 ± 3.07	9,160 ± 4,656	2.70 ±0.01	8,878 ± 1,045	2.28 ±0.14
High Analytical	10,697 ±10,574	8.72 ± 7.59	35,996 ±24,050	11.54 ± 2.08	12,011	5.53[d]	803 ± 751	0.68 ±0.30
High Technical	49,454 ±22,421	33.69 ±20.64	24,558 ±14,815	11.31 ± 4.80	12,129 ± 7,539	6.64 ±4.06	N.S.	N.S.[e]

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose for 43 days.

[b] Values represent net counts per minute (MCPM) ±SE.

[c] Values represent isotope incorporation index (III) ±SE.

[d] n = 1.

[e] N.S. = Insufficient tissue recovered due to treatment.
N.D. = Not determined.
No significant differences found (P>.05) for blood, lymph node or spleen.

primary and secondary antibody response following IV immunization with HRBC. Changes in immunoglobulin levels over the last 10 days of pentachlorophenol exposure are shown in Figure 2.1. Although reductions in all three immunoglobulins measured are readily apparent in the high technical group, no statistical differences ($P > .05$) could be detected. The primary and secondary *in vivo* responses to HRBC (Figure 2.2) were similar for all groups in both mean peak titer and kinetics as measured by hemagglutinating antibody.

Neutrophil function was elevated on the last day of PCP exposure by chemiluminescent responses after *in vitro* challenge with opsonized zymosan (Figure 2.3). The chemiluminescent responses of neutrophils from control and PCP-treated calves were not significantly different in either peak height or kinetics. However, it is interesting to note that the peak response occurred five minutes earlier using control PMNs compared to PMNs from any of the PCP-treated groups.

Histology

Gross evaluation of spleen, liver, and kidney revealed a statistically significant ($P < .01$) increase in liver weight in both technical pentachlorophenol groups, and an increase ($P < .05$) in kidney size in the high dose tPCP group (Table 2.7). No functional impairment was evident in the kidneys as indicated by clinicopathologic results shown in Tables 2.2 and 2.3.

The change in the thymus weights was most remarkable (Table 2.7). The mean calf thymus weight from the high dose

Table 2.7. Organ Weights of Calves Fed Analytical or Technical Grade Pentachlorophenol[a]

Experimental Group	n	Thymus Wt(g)[b]	Thymus %Br[c]	Thymus %Bo[d]	Spleen Wt(g)	Spleen %Br	Spleen %Bo	Liver Wt(g)	Liver %Br	Liver %Bo	Kidney Wt(g)	Kidney %Br	Kidney %Bo	Weight Brain (g)	Weight Body (kg)
Control	3	152 ±18	56	0.26	183 ±19	67	0.31	1183 ±73	435	2.0	300 ± 12	110	0.51	273 ± 9	59 ± 2
Low Analytical	3	159 ±45	58	0.29	141 ±21	52	0.26	1142 ±126	427	2.1	288 ± 51	107	0.53	267 ±17	54 ± 8
Low Technical	3	93 ±39	34	0.17	193 ±44	71	0.35	1480 ±106	542	2.7[e]	348 ± 7	128	0.63	273 ± 7	55 ± 5
High Analytical	2	71 ±49	25	0.13	125 ±45	44	0.22	1218 ±273	426	2.2	345 ± 55	121	0.62	285 ± 5	56 ±10
High Technical	2	26 ± 4	10	0.05[f]	88 ± 7	34	0.18	1335 ± 86	520	2.7[e]	345 ± 25	134	0.70[f]	260 ±40	49 ± 4

[a] 1.0 mg/kg body wt/day = low dose, 10 mg/kg body wt/day = high dose, for 43 days.

[b] Mean organ weight (wt) in grams ±SE.

[c] Organ weight as percent of brain (Br) weight.

[d] Organ weight as percent of body (Bo) weight.

[e] Significantly different from control ($P<0.01$).

[f] Significantly different from control ($P<0.05$).

technical group decreased to 17% of controls. Histologic examination of a thymus section from a calf in the high dose tPCP group (10 mg/kg/day) after 42 days of exposure revealed that the atrophy in the tPCP exposed thymus was a result of an absence of cortical lymphocytes. Cortical lymphocytes were readily apparent in the control thymus. Spleen weights were reduced by PCP exposure, but not significantly. No other significant histological changes associated with pentachlorophenol exposure were observed.

DISCUSSION AND CONCLUSIONS

Toxic effects from PCP were more evident at the higher of the two doses (10 mg/kg) and in the calves fed tPCP versus aPCP. The total steady state (acid hydrolyzed) pentachlorophenol concentration in the blood of the 1.0 mg/kg and 10.0 mg/kg groups was 32 ppm and 92 ppm, respectively. There were no significant differences in blood levels reached between the two grades of pentachlorophenol used.

The thymus, spleen, bone marrow, lymph, liver and kidney contained 21.6, 7.4, 12.5, 13.4, 25.3, and 26.6 ppm PCP, respectively, in the 10 mg tPCP/kg treatment group. At the 1.0 mg tPCP/kg level, these tissues contained 3.5, 2.3, 1.6, 4.5, 6.2, and 7.2 ppm, respectively. As was the case with the blood levels, PCP in these tissues after exposure to aPCP was roughly equivalent to those occurring after tPCP exposure.

Total protein and albumin values dropped as a result of pentachlorophenol exposure and were lowest in the 10 mg/kg tPCP group. These declines may not be directly associated with PCP exposure. The calves in each group were not pair fed (see Materials and Methods). During the time grain was fed, calves in the 10 mg tPCP/kg group consumed only 14% of the grain consumed by the control calves, and calves in the 10 mg aPCP/kg group consumed 67% of the control calf grain intake. All other groups were on a near equal grain consumption basis with the control calves. As a result, the calves in the 10 mg/kg tPCP group weighed an average of 10 kg or 17% less than control

calves at the end of the six weeks. The poor grain intake could well have been the sole contributing factor responsible for the decreased total protein and albumin levels, particularly since protein was not lost in the urine.

The substantially elevated gamma-glutamyl transferase (GGT) activity which increased in a dose-response fashion with pentachlorophenol exposure, especially with the technical grade PCP, is associated with the hepatomegaly observed in both the 1.0 mg/kg and 10 mg/kg tPCP groups. Typically, GGT is an indicator of hepatobiliary disease and its activity follows that of alkaline phosphatase (ALP), or more specifically, the hepatic isoenzyme of ALP. In this case, no increase was seen in ALP or any of the other indicators of hepatobiliary disease. Since GGT is a microsomal enzyme and its level increases in response to microsomal enzyme induction, and since it is known that contaminants in technical pentachlorophenol are inducers of mixed function oxidases in cattle (McConnell et al., 1980); the rising GGT level correlated very well with microsomal enzyme induction and increased liver weights.

The earliest clinical sign of pentachlorophenol toxicity in cattle, as reported by McConnell et al.,(1980) is the presence of a progressive normocytic, normochromic anemia. Cattle in the McConnell study had been exposed to 20 mg/kg/day of pentachlorophenol containing various levels of technical pentachlorophenol. The anemia was first detected at week four and was attributable to the toxic impurities in

pentachlorophenol. In the present study, no anemia was evident at the end of the six weeks. Similarily, in the study with lactating dairy cows in which they were dosed with tPCP at 0.2 mg/kg/day for 75 to 84 days followed by 2.0 mg/kg for 56 to 62 days, no clinical signs of anemia were present. Evidentally, high levels of exposure to pentachlorophenol and its toxic contaminants over periods of many weeks is required in order to produce clinical signs of anemia in cattle.

The data is this study show that thymic atrophy occurs long before anemia would become evident and at much lower levels of tPCP exposure. The effect of 10 mg/kg tPCP treatment was so devastating on the thymus tissue of these calves that it was not possible to isolate adequate numbers of lymphocytes from these tissues for the immunologic profile. Interestingly, those lymphocytes that were obtained from thymic tissue contained En markers (T cell markers) in the same concentration as thymocytes from the control animals. Evidently, whatever is causing the cortical depletion of lymphocytes from the thymus is not selectively affecting either the En set or null set of lymphocytes present.

The degree of thymic atrophy in the 10 mg/kg aPCP group while dramatic, was not quite as marked at it was in the thymic tissue from calves fed 10 mg/kg tPCP. Yet, the blastogenic response was already severely reduced in the 10 mg/kg aPCP calves. The reduced blastogenic response seems to precede atrophy, but could not be detected in the blood stream,

spleen, or lymph node. Reduced responses by thymocytes to mitogens has previously been observed in rats (Vos and Moore, 1974) and mice (Vos et al., 1978) exposed to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). Although tPCP does not contain 2, 3, 7, 8-TCDD, it does contain a small amount of other tetrachlorodibenzo-p-dioxin isomers (Kinzell et al., 1981) and a significantly larger amount of other highly chlorinated congeners. These congeners can act within an animal species similar to 2, 3, 7, 8-TCDD if a greater exposure is used (McConnell et al., 1978).

Stress is known to cause thymic atrophy mediated via increased production of adrenal glucocorticoid hormones (Dougherty et al., 1964). However, elevated cortisol (hydrocortisone) levels did not cause thymic atrophy in these calves since all calves tested had low normal cortisol levels (Table 2.3).

Other measurements of immune function resulted in no immunomodulation detected due to PCP treatment. These data indicate that no immunologic effect was detected in either the 1.0 mg/kg aPCP or tPCP exposed calves and is consistent with the findings in mature lactating dairy cattle in which no quantitative or functional immunologic defects were seen when these cattle were exposed to up to 2.0 mg/kg tPCP (See Chapter 1). This is also consistent with the results reported by McConnell et al. (1980) in which no changes were seen in the immune system of non-lactating heifers given 1.5 mg/kg tPCP plus 13.5 mg aPCP/kg/day;beyond that which was

observed with 15 mg/kg aPCP. In this study, calf performance was poor at the 10 mg/kg level yet effects on the immuns system were minimal. Quantitative effects were limited to thymic atrophy. The only functional impairment was the decreased mitogen-induced blastogenic response in thymocytes. In comparison, McConnell et al., (1980) reported a decrease in thymus weight and an enhancement of blastogenesis in blood derived lymphocytes as the only immune related changes attributable to exposure to the contaminants in 5.25 and 15 mg tPCP/kg/day.

In summary, immunomodulation did not occur at levels of PCP exposure (1.0 mg/kg/day) which resulted in blood PCP levels of 32 ppm. At higher levels of exposure (10 mg/kg/day), PCP is immunomodulating as evidenced by the effects on the thymus. However, most of the effects are seen in and are associated with the contaminants present in the technical grade PCP.

Chapter 3

BOVINE NEUTROPHIL SHAPE CHANGE KINETICS AND ITS INHIBITION BY PENTACHLOROPHENOL

INTRODUCTION

Polymorphonuclear leucocytes (PMNs) are phagocytic cells intricately involved in host defense. In recent years, the identification of cell surface receptors on PMNs for immunoglobulins, complement components, and other chemotactic factors have advanced our understanding of the cellular signals which influence the chemotactic and phagocytic activities of these cells. Many additional chemotactic factors are known (Wilkinson, 1974). In 1979, Smith et al. studied the initial morphological changes in neutrophils immediately after exposure to chemotactic factors. These investigators noted that chemotactic factors induce a characteristic morphologic change--a bipolar form. Agents which have no chemotactic activity do not alter the morphologic features of neutrophils.

Information regarding the chemotactic response of bovine neutrophils is limited. Only two techniques have been used to evaluate neutrophil chemotaxis in cattle, the Boyden chamber and migration under agarose techniques (Roth and Kaeberle, 1981a, 1981b, and Markham et al., 1982). This report describes a rapid, inexpensive assay to evaluate chemotaxis in bovine neutrophils which utilizes the change in neutrophil shape from spherical to bipolar forms. Several agents which are chemotactic for human neutrophils were also chemotactic for bovine neutrophils. Interestingly, however, f-Met-Leu-Phe, a potent chemotactic agent for human neutrophils, was totally inactive for bovine neutrophils.

In addition, three different grades of pentachlorophenol were tested for their influence on neutrophil chemotaxis. These studies indicate that PCP could inhibit the neutrophil shape change response at concentrations of 0.1 mg/ml. Perhaps the neutrophil shape change response may be a useful assay in screening agents of environmental concern for their influence on a primary host defense system.

METHODS AND MATERIALS

Isolation of Bovine Neutrophils

Blood samples from healthy, mature and lactating Holstein-Friesian cattle were collected in sodium citrate. Neutrophils were obtained by either centrifugation of whole anticoagulated blood or centrifugation on Ficoll-Hypaque (Ficoll Pharmacia Fine Chemicals Inc., Piscataway, NJ; Hypaque-Sterling Organics, NY) cushions (Boyum, 1968). The neutrophils recovered from anticoagulated whole blood were isolated after centrifugation at 600x g for 20 minutes. The plasma and one-third of the packed red blood cell (RBC) volume were removed and discarded. To the remaining RBC pellet, two volumes of sterile, cold, double-distilled water (DDW) were added. The hypotonic lysis of erythrocytes continued for one minute, and was stopped by the addition of one volume of three times the physiologic concentration of NaCl (2.7%) in sterile DDW. After erythrocyte lysis, leucocytes were washed three times at 200 x g for seven minutes with Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution (HBSS). A second hypotonic shock was required to remove residual RBCs in some leucocyte preparations. The leucocytes obtained were at least 99% granulocytes. Of these, approximately 80-95% were neutrophils (PMN) and the remainder were eosinophils. Red blood cells were absent and platelets were few in these preparations.

Polymorphonuclear leucocytes isolated from Ficoll-Hypaque (F/H) cushions were obtained following centrifugation at 900 x g for 40 minutes. The plasma, mononuclear cells and all but

the packed RBC layer were removed and discarded. The cell pellet was then treated by hypotonic shock as described above. Yields were slightly lower than those obtained by the centrifugation of whole blood.

The viability of all neutrophil suspensions was at least 99% as determined by trypan blue dye exclusion. Neutrophil suspensions were adjusted to 10^7 cells/ml in HBSS for shape change studies.

Assessment of Neutrophil Shape Changes

All glassware for these studies was treated with a mixture of three parts PBS and one part autologous serum. A 0.1 ml aliquot of the neutrophil suspension was diluted in 0.9 ml of phosphate buffered saline (PBS). The cells were then exposed to various conditions and biological and chemical substances to evaluate neutrophil shape change. Neutrophils undergoing shape changes were terminated by the dropwise addition of 2-3 ml of 1% glutaraldehyde (Sigma Chemical Co.) in PBS. The neutrophil suspensions were mixed constantly while glutaraldehyde was added. The PMN glutaraldehyde mixture was then allowed to stand overnight at $4^{\circ}C$. Cells were examined using 1000X phase-contrast microscopy and classified according to shape using the classification system of Smith et al.,(1979).

Kinetic studies were performed at $37^{\circ}C$ using 0.1 ml of 10^7 PMN/ml added to 0.9 ml PBS containing zymosan-activated serum (ZAS). The ZAS was prepared by incubating 10 mg zymosan (Sigma Chemical Co.) in 1.0 ml fresh bovine serum for 45 minutes

at 37°C. The serum was cleared by centrifugation and diluted with PBS before addition of PMNs.

Once shape change parameters were standardized using ZAS, other biological and chemical agents reported to cause shape change in human PMNs were investigated using bovine neutrophils. The agents tested included N-formyl-L-methionyl-L-leucinyl-L-phenylalanine (f-Met-Leu-Phe)(Andrulis Research Corp., Bethesda, MD)(Schiffmann et al., 1975),alpha-casein, and hydrolyzed and partially dephosphorylated casein (Sigma Chemical Co) (Wilkinson, 1972) and bacterial cell filtrate (BCF) (Keller and Sorkin, 1967). F-Met-Leu-Phe was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10^{-3} molar (M), then diluted to concentrations of 10^{-5}, 10^{-7}, and 10^{-9} M in PBS. Casein and alpha-casein were dissolved in HBSS and adjusted to pH 7.2. Final concentrations of these caseins in cell suspensions ranged from 0.1 to 25 mg/ml. Bacterial cell filtrates from E. coli ATCC #25922 (Difco Laboratories, Detroit, MI) were prepared in HBSS. Bacteria were cultured for 24 to 48 hours at 37°C. The cell suspension was then filtered through a 0.22-um Millipore filter and the pH of the culture was adjusted to 7.4. The filtrate (BCF) was tested at concentrations ranging from full strength to 0.1% in HBSS.

Influence of Pentachlorophenol (PCP) on Neutrophil Shape Change

These studies utilized three grades of PCP: analytical grade PCP (aPCP) (Aldrich Chem. Co.,Milwaukee,WI) purified PCP(pPCP) (Dow Chem. Co., Midland, MI) and technical PCP(tPCP)(a commercial pentachlorophenol which was generously provided by the American

Wood Preservers Institute). The tPCP represented an industry composite from the three major manufacturers of pentachlorophenol.

Each grade of PCP was dissolved in either ethyl alcohol (ETOH) or DMSO to a concentration of 20 mg/ml. Various concentrations of the PCP solutions (0.2 mg/ml to 0.02 mg/ml) were used in the neutrophil shape change studies. In some experiments, PCP solutions were added to neutrophil suspensions incubated in 1% ZAS. The PMN shape changes were assessed after a seven minute reaction time.

RESULTS

Effects of Chemotactic Factors on Cell Shape Change

The appearance of unstimulated PMN and eosinophils separated from anticoagulated blood is shown in Figure 3.1a. The cells appear round, have a centrally located nucleus and have a slightly ruffled membrane on a small portion of the surface. Incubation of neutrophil suspensions in 1% ZAS caused significant shape changes within two to three minutes. At least 50% of the PMN were elongated with a few showing uropod formation. To quantify the shape change response, the cells were categorized as spherical or nonspherical. The spherical classification included round cells and cells whose shape was generally round with a ruffled membrane (Figure 3.1b). The nonspherical category included cells whose overall form was elongated (bipolar) with, or without uropods (Figure 3.1c). The neutrophil shape change response to various doses of ZAS is presented in Figure 3.2. The time course for shape change after addition of 1% ZAS in PMN suspensions is presented in Figure 3.3.

Neutrophils isolated from Ficoll/Hypaque cushions also changed shape in response to ZAS. However, this PMN isolation procedure produced a higher percentage of dead cells, as well as cells showing "abnormal" shapes and excessive membrane ruffling in unstimulated control experiments. Neutrophils recovered from Ficoll-Hypaque cushions were less responsive to 1% ZAS in that only 25 to 40% of the cells exhibited chemotactic factor-induced shape changes.

Figure 3.1a. Bovine Neutrophils (N) and Eosinophils (E) (x 1,000 Phase Contrast) Fixed in 1% Glutaraldehyde After a Five Minute Incubation at 37°C with no CF. The Bar at the Bottom Left Represents 10 Microns.

Figure 3.1b. Bovine Neutrophils (N) Showing Round Ruffled Features (Spherical Classification). Cells Were Fixed in 1% Glutaraldehyde and Photographed at x 1,000 Using Phase Contrast. One Nonstimulated Eosinophil (E) is present.

N
E

N
E

Figure 3.1c. Bovine Neutrophils (x 1,000 Phase Contrast) Showing Nonspherical (Bipolar) Forms With One Neutrophil Showing the Beginning of a Uropod (U). Cells Were Fixed With 1% Glutaraldehyde After Five Minutes Incubation at 37°C in the Presence of 1% ZAS.

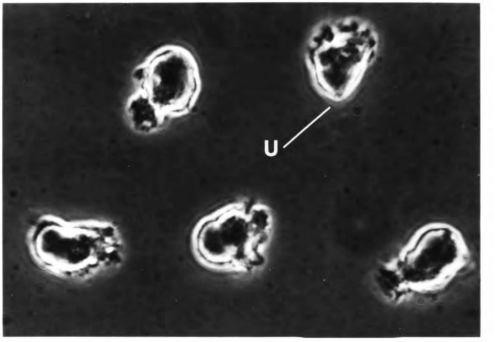

U

Figure 3.1d. Bovine Eosinophil (E) Showing a Typical Response to 1% ZAS (x 1,000 Phase Contrast). A Bipolar Neutrophil (N) is Also Present.

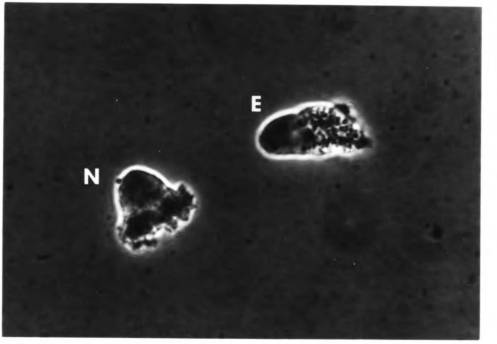
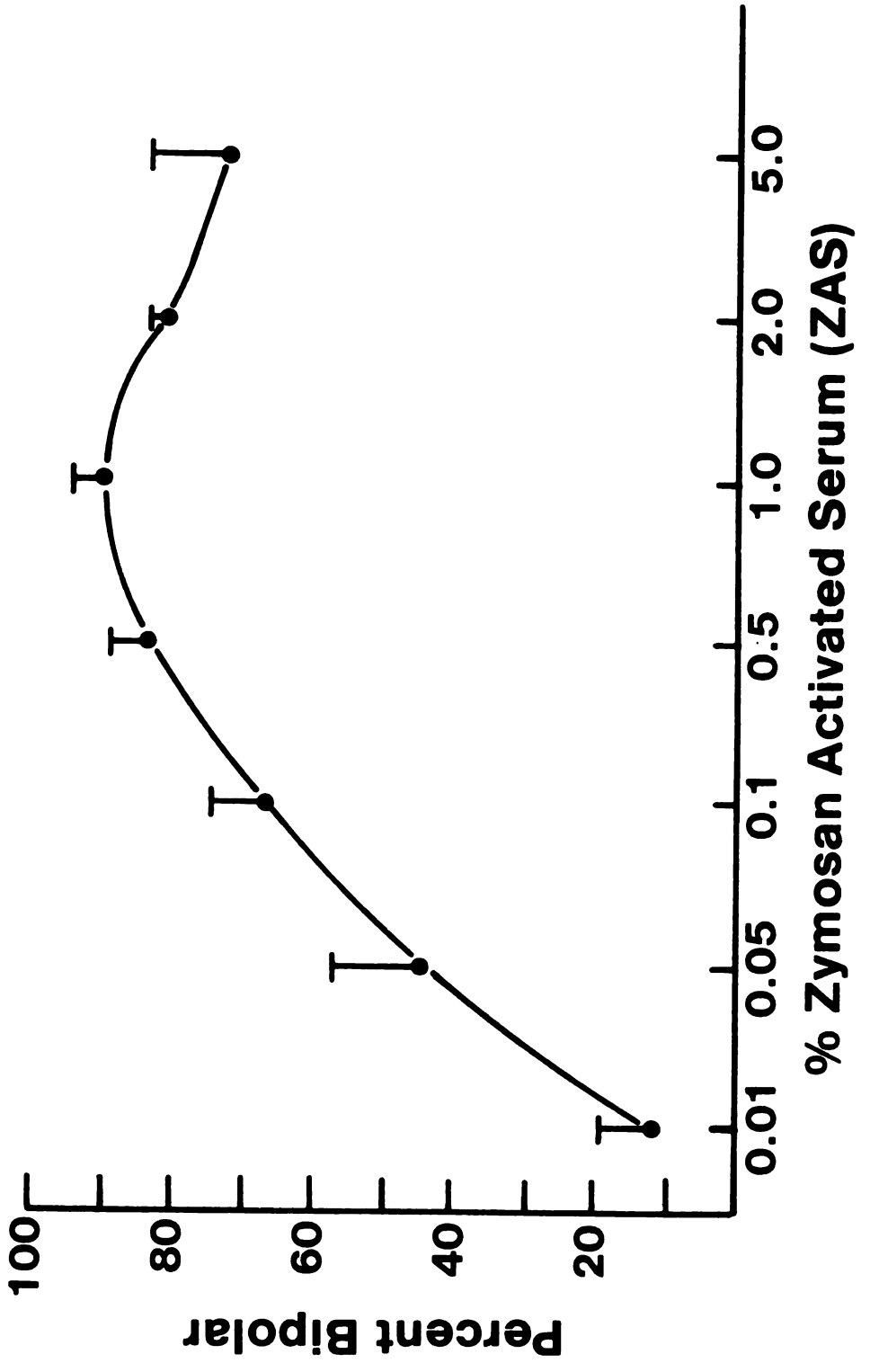

E
N

Figure 3.2. Change in Shape of Neutrophils Exposed to Various Levels of ZAS. "Percent Bipolar" Refers to the Percent of Cells in the Suspension with a Bipolar Shape (Bipolar Plus Uropod). Incubation Time was Five Minutes. Each Point is the Mean $\pm$ of Four Separate Experiments.


Percent Bipolar
100
80
60
40
20
0.01
0.05
0.1
0.5
1.0
2.0
5.0
% Zymosan Activated Serum (ZAS)

Neutrophils incubated with casein and three casein-derivatives were evaluated for shape changes over a ten minute interval. The casein and alpha-casein response is shown in Figure 3.4. The response to casein is less dramatic than the response to ZAS. Also, the maximum shape change caused by casein was often observed to occur together with cell morphologic alterations similar to those preceding cell death. Acid hydrolysate type I casein and a vitamin free casein were able to stimulate shape change, but to a much lesser extent than either the alpha-casein or the partially dephosphorylated and hydrolyzed casein.

Filtrates of E. coli cultures (BCF) also induced shape changes in bovine neutrophils. Undiluted BCF was most active, while BCF diluted 1:4 in HBSS was ineffective in inducing shape changes. It is noteworthy that BCF preparations varied significantly. Some preparations stimulated shape changes in at least 60% of PMN while other preparations only induced shape change in 25% of PMN.

The peptide f-Met-Leu-Phe was ineffective in causing shape changes in bovine neutrophils over a wide concentration range. At the "most optimum" concentration of 10^{-7}M, f-Met-Leu-Phe stimulated only 1.5-3.5% of PMN into the nonspherical classification.

Eosinophils contaminating neutrophil preparations also responsed to ZAS. They could be stimulated to a much greater extent than were PMN when incubated with 1% ZAS (Figure 3.1d).

Figure 3.3. Change in Shape in Neutrophils Exposed to 1% ZAS. "Percent Bipolar" Refers to the Percent of Cells in the Suspension with a Bipolar Shape (Bipolar Plus Uropod). "Time-minutes" Refers to the Time After the Addition of Cells. Each Point is the Mean $\pm$ SE for Four Separate Experiments.

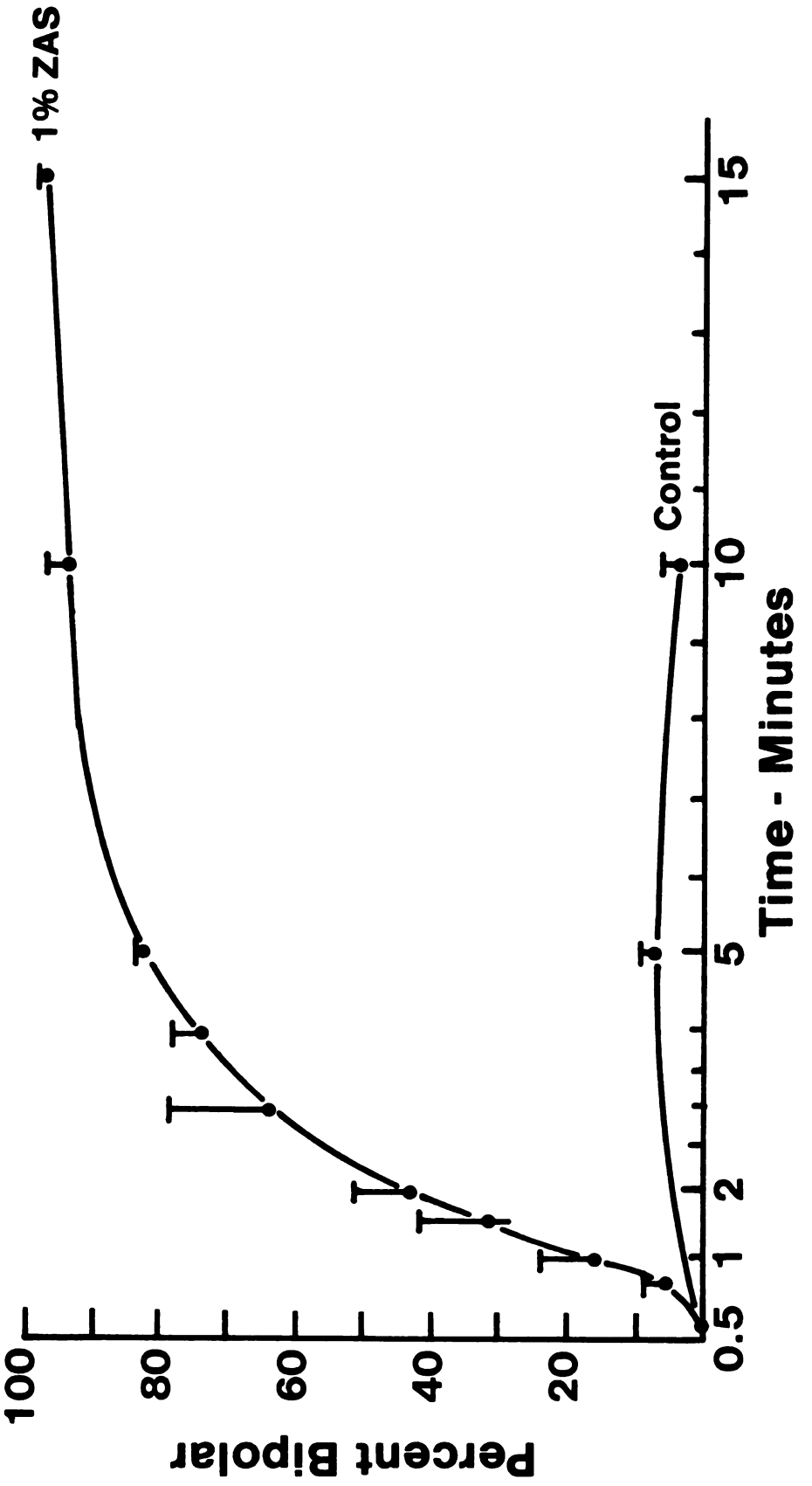
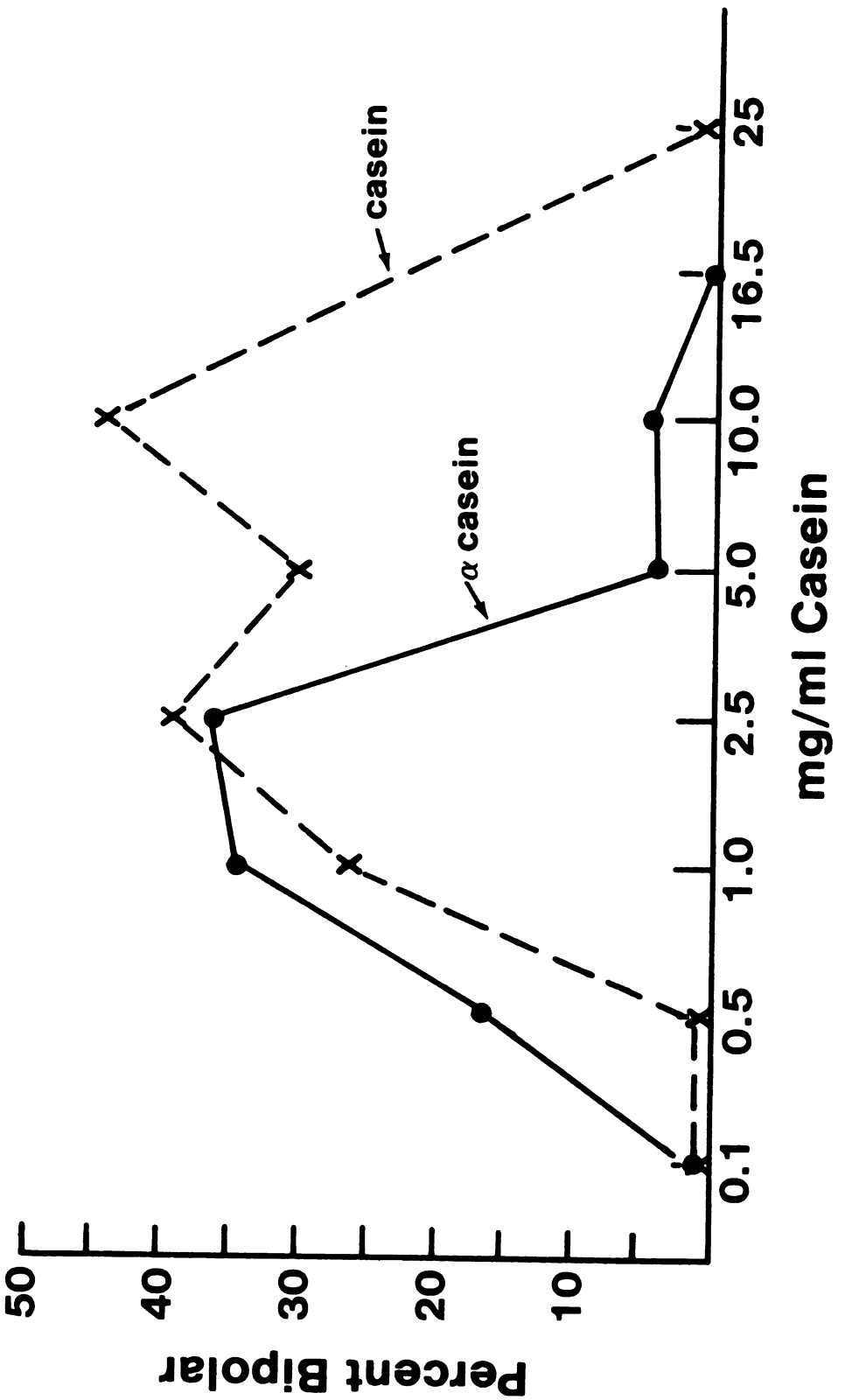

1% ZAS
Control
Percent Bipolar
100
80
60
40
20
0.5
1
2
5
10
15
Time - Minutes

Figure 3.4. Change in Shape of Neutrophils Exposed to Various Levels of Alpha-casein and Casein (Partially Dephosphorylated and Hydrolyzed). "Percent Bipolar" Refers to the Percent (Mean) of Neutrophils in the Suspension with a Bipolar Shape (Bipolar Plus Uropod). Incubation Time was 7.5 Minutes.


Percent Bipolar
50
40
30
20
10
0.1
0.5
1.0
2.5
5.0
10.0
16.5
25
mg/ml Casein
casein
α casein

It is noteworthy, however, that eosinophils did not change shape when exposed to either BCF or casein.

Effects of Pentachlorophenol on Cell Shape Change

Results of studies evaluating the influence of aPCP, tPCP, and pPCP on bovine neutrophil shape changes are presented in Figure 3.5. All three grades of PCP significantly reduced shape-change responses in PMNs exposed to 1% ZAS. The shape change response could be eliminated at 0.1 mg/ml PCP. No shape changes were induced in neutrophils incubated with any of the three grades of PCP, or the two solvents, DMSO or ETOH. Neither was DMSO or ETOH able to inhibit shape change when PMN were incubated with ZAS in the absense of PCP.

A representative example of ZAS stimulated PMNs exposed to PCP is presented in Figure 3.6. These "wrinkled" neutrophils were able to exclude trypan blue for ninety minutes while at 37^{o}C and in the presence of any of the three PCPs at 0.2 mg/ml. Significant numbers of dead cells began to appear at two hours. Solvent controls showed at least 98% viability at two and one-half hours.

Figure 3.5. Change in Shape of Neutrophils Exposed to 1% Zymosan Activated Serum in the Presence of Three Different Grades of Pentachlorophenol (PCP); Analytical or aPCP, Purified or pPCP and Technical or tPCP. The Numbers Above the Bars Refer to mg PCP/ml Used. The Closed Bars Refer to Ethanol and the Open Bars to Dimethyl Sulfoxide Used as Solvents to Deliver the PCPs. "Percent Bipolar" Refers to the Percent of Cells in the Suspension with a Bipolar Shape (Bipolar Plus Uropod). Incubation Time was 7.5 Minutes. The Controls Contained 1% Solvent, the Maximum Used to Deliver PCP.

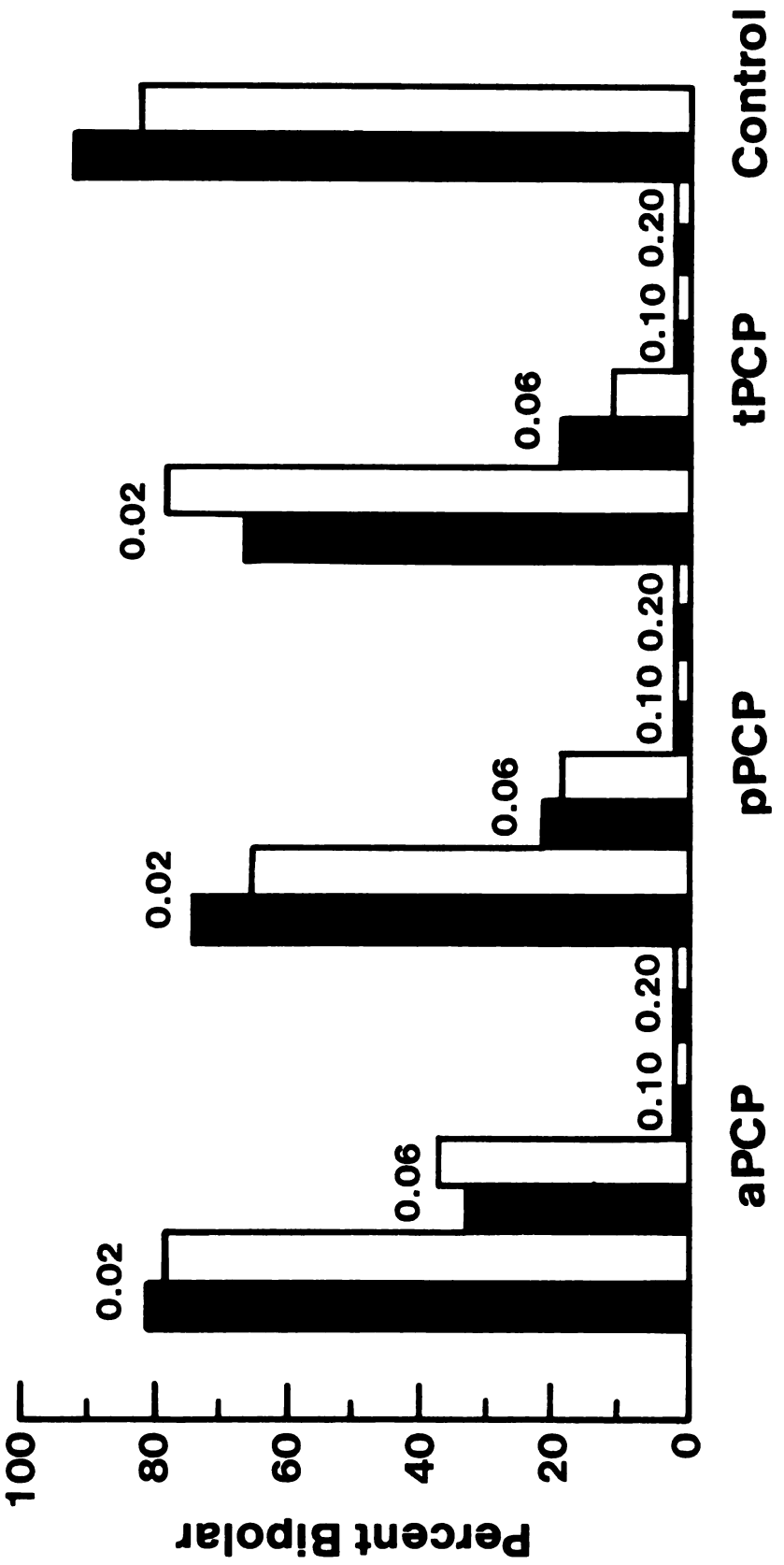
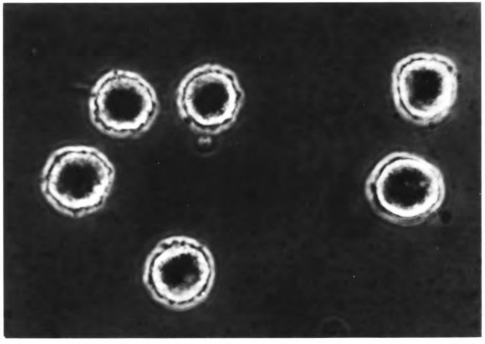

Percent Bipolar
100
80
60
40
20
0
0.02
0.06
0.10
0.20
aPCP
pPCP
tPCP
Control

Figure 3.6. Bovine Neutrophils Showing the Unresponsive "Wrinkled" Appearance of Cells Incubated in the Presence of 0.10 mg/ml PCP and 1% ZAS for 7.5 Minutes at 37°C (x 1,000 Phase Contrast).



DISCUSSION AND CONCLUSIONS

This study shows that bovine neutrophils respond to many of the same chemotactic factors as human neutrophils. However, bovine PMN were totally unresponsive to f-Met-Leu-Phe, which is a potent chemotactic factor for human neutrophils (Schiffmann et al., 1975).

One percent ZAS was found to be the optimum concentration for neutrophils to change shape. It is noteworthy that a ZAS concentration greater than two percent inhibited the bipolar response, a phenomenon that has been reported in human PMN (Keller and Sorkin, 1966). Bovine neutrophils, however, appear to be more sensitive since they are inhibited at a lower ZAS concentration than that required for the inhibition of human neutrophil chemotaxis (greater than 10%) (Smith et al, 1979).

Casein and bacterial metabolites (Keller and Sorkin, 1965) are known to be chemotactic for human neutrophils. Similar results were noted for bovine neutrophils. Russell and Reiter (1975 and 1976), however, have reported that casein inhibits both ingestion and intracellular killing of bacteria by bovine neutrophils. The inhibition occurred at physiological concentrations of native casein micelles, and by the addition of purified casein as sodium caseinate. These data indicate that casein may have significant effects on neutrophil function which occur below the physiologic range of casein in milk (25-40 mg/ml). Alpha-casein at 5 mg/ml and hydrolysed casein at 10 to 25 mg/ml caused a significant decrease in the neutrophil shape change response. This impairment in neutrophil shape change probably precedes and is related to the casein-induced decrease in

PMN phagocytosis and bactericidal activity observed by Russell and Teiter (1975). Such impairment in neutrophil function by casein may enhance the susceptibility of the udder to bacterial infection.

It is interesting that casein and BCF will not cause appreciable shape change in the bovine eosinophil. In contrast, the eosinophil appears to respond faster, and to a greater degree than PMN to ZAS. These date suggest differences in receptors or receptor density on their cell surfaces.

Of particular interest was the significant effect that Ficoll-Hypaque gradient separation had on the bipolar shape change response. Whereas Ficoll-Hypaque cushions and heparinized blood appear satisfactory for the isolation of bovine lymphocytes for subpopulation enumeration and function studies, (see previous chapters), their use markedly inhibits shape change in neutrophils. Heparinized blood caused neutrophil clumping, and the preparations were significantly comtaminated with platelets. Others have noted difficulties associated with the use of F/H cushions for separating bovine neutrophils for use in in vitro studies. Roth and Kaeberle (1981) reported that granulocytes exposed to F/H had significantly reduced random migration under agarose while other granulocyte function tests such as chemiluminescence were not significantly altered. Lichtman et al., (1976) reported the presence of stimulated neutrophil shapes when human cells were maintained in heparinized plasma. In addition, use of F/H cushions with or without heparinized samples resulted in less

PMN shape change in response to CF. Thus, neutrophils from both humans and cattle may be functionally impaired due to contact with Ficoll/Hypaque gradients and heparinized blood.

An implied association between exposure to penta and poor health in dairy cattle was reported by Thomas et al., (1975). Clinical signs in the cattle included decreased milk production, poor general appearance, skin lesions, increased mastitis, persistent infections, high calf mortality and death. Some of these effects are suggestive of a suppression in host defense.

In a previous study, the effect of technical grade pentachlorophenol upon lactating dairy cattle performance, general health, histology (Kinzell et al., 1981) and immune function was evaluated. The level of technical pentachlorophenol exposure used in that study was 0.2 mg/kg/day for 75-84 days followed by 2.0 mg/kg/day for an additional 56-62 days. The maximum level of PCP reached in blood during these studies was 0.014 mg/ml. This level is similar to the lowest dose used in the present in vitro study, a level of PCP in which no significant impairment in shape change response was seen.. The importance of this finding is that technical pentachlorophenol administered subchronically in the previous study was designed to approximate a level of exposure which dairy cattle might be expected to contact in a farm environment (Van Gelder, 1977). No immunomodulation could be detected resulting from technical pentachlorophenol exposure in that study (Chapter 1).

The observation that chemiluminescence by neutrophils exposed to PCP always peaked five minutes later than control neutrophils was puzzling (Chapter 1), but was regarded as not important at that time. Since that study, I have made this observation again in calves exposed to PCP (Chapter 2). It is interesting to speculate whether this phenomenon is related to the inhibition of shape change seen *in vitro*.

At levels one to two orders of magnitude higher, pentachlorophenol in the blood can reach 0.1 mg/ml (Parker *et al.*, 1980). This blood level of PCP has been reached in a study done in nonlactating cattle (McConnell *et al.*, 1980). It showed (among a lot of negative findings) an enhancement of mitogen-induced lymphoblastogenesis and a decrease in absolute and relative thymus weight, both of which were attributed to the amount of contaminants in the pentachlorophenol fed. No effect was seen on white blood cell number or differential leucocyte count. At the level of PCP exposure used in that study, a level which could be reached in an acute exposure, there data indicate that an effect on bovine neutrophils could occur. That an effect could occur at this level is also supported by the fact that locomotion and perhaps shape change requires some oxidative energy (Carruthers, 1967), and pentachlorophenol is an uncoupler of oxidative phosphorylation. Carruthers found that dinitrophenol, an uncoupler of oxidative phosphorylation, at a concentration of 1×10^{-3}M or 0.184 mg/ml had a partial inhibitory effect on both random and directed motility of human leucocytes *in vitro*. *In vivo* investigation of this phenomenon should reveal

interesting results.

The data presented above provide the framework for further studies involving many aspects of bovine eosinophil and neutrophil investigations; especially in the areas of chemotaxis, shape change, adhesiveness, motility and chemotactic factors.

In summary, the data presented define the parameters for performing the isolation and analysis of shape change in bovine neutrophils. These data can be used as a basis to investigate other areas of bovine neutrophil function.

Chapter 4

ISOLATED BOVINE HEPATOCYTES:

A MODEL FOR XENOBIOTIC METABOLISM

INTRODUCTION

Isolation of viable rat hepatocytes with collagenase was first described by Berry and Friend in 1969. In 1972 and 1973, Seglen published detailed descriptions of the factors required for optimizing the isolation of rat hepatocytes. Seglen was the first to perform the two-step method which has formed the basis for all isolation procedures. The isolation of rat hepatocytes has since become a routine procedure in many laboratories.

The thrust of isolated hepatocyte research is in two major areas: in expanding the number of applications for isolated rat cellsand in establishing long-lived cultures of hepatocytes which do not dedifferentiate. Recently, many researchers in toxicology have changed from systems based on liver subcellular fractions, organ perfusions, or slices,to isolated hepatocytes. This change occurred because isolated hepatocytes seem to mirror the *in vivo* metabolism of xenobiotics more closely than other *in vitro* systems through the accountability of structure and kinetics (Fry and Bridges, 1977; Menzer, 1979).

No reports describe the isolation of hepatocytes from cattle, although hepatocytes have been isolated from non-laboratory animal species such as sheep (Clark *et al.*, 1976 ; Ash and Pogson, 1977) and humans (Liddiard *et al.*, 1980 ; Reese and Byard, 1981; Strom *et al.*, 1982). Since cattle are often too large and expensive for use in toxicokinetic studies, isolated bovine hepatocytes would be an ideal alternative.

This paper describes an approach that can be used to prepare isolated hepatocytes from bovine liver. The isolation procedure results in large numbers of viable hepatocytes which are metabolically active, and can be used for metabolizing xenobiotics.

MATERIALS AND METHODS

Reagents

Perfusion (Ca^{2+}/Mg^{2+}-free) buffer: 8.3 g sodium chloride, 0.5g potassium chloride, 2.4 g HEPES buffer and 0.1mM ethylene-bis (oxyethylene-nitrilo) tetraacetic acid (EGTA) were dissolved in 1 liter of double-distilled water, and adjusted to a final pH of 7.4 with 1M NaOH. Just prior to use, the solution was saturated with 95% O_2/5% CO_2.

Collagenase Solution: prepared by the addition of 5mM calcium chloride and 50 mg/dl collagenase[1] to the perfusion buffer. The collagenase was added just before use.

Wash buffer: same composition as the perfusion buffer, but without EGTA.

Trypan blue solution: a filtered solution of 0.4% trypan blue in normal saline.

Gluconeogenesis medium: Earle's balanced salt solution free of glucose and phosphate and fortified with 20mM lactic acid, 4mM lysine, 2mM pyruvate, 20mM sodium acetate, 0.2mM dibutyryl cAMP. The pH was adjusted to 7.45 with sodium hydroxide. Control gluconeogenesis medium was the same as the gluconeogenesis medium but without the addition of 20mM lactic acid, 4mM lysine and 2mM pyruvate.

Palmitic acid oxidation media: Calcium-free Krebs-Ringer

[1]Collagenase II was obtained from Worthington Biochemical Corp., Freehold, NJ and Type I Collagenase from Sigma Chemical Co., St. Louis, MO.

bicarbonate containing 1mM-^{14}C-palmitate[2] and 2mM DL-carnitine. The palmitate was complexed with 1.5% bovine serum albumin at a 4:1 fatty acid to bovine serum albumin ratio. The final pH was adjusted to 7.4 with CO_2.

Homogenization buffer for cytochrome P-450: 20mM Tris-hydrochloric acid, 1.15% potassium chloride, pH 7.4; 2.4g Trizma base and 15g potassium chloride were dissolved in approximately 800 ml of double distilled water, the pH adjusted to 7.4 using 100mM hydrochloric acid, and brought to a volume of one liter with double-distilled H_2O.

Isolation of Bovine Hepatocytes

Livers were generally from mature Holstein-Friesian dairy cattle but were occasionally from calves and steers of other breeds. Preliminary experiments revealed that the caudate process of the bovine liver was best for preparation of viable hepatocytes. Viability was not affected if the caudate process was removed within 15 min following euthanasia.

The caudate process was removed by cutting across its base of attachment. The cross sectional area of the cut surface was kept to a minimum. Immediately after removal, the caudate process was perfused with approximately 300 ml of cold perfusion buffer. This was accomplished by inserting a plastic syringe tip of a 50 ml syringe into a number of the larger exposed arteries and veins on the cut face and repeatedly perfusing

[2] 1-^{14}C-Palmitate was obtained from Amersham, Arlington Heights, IL.

until the tissue was uniformly blanched.

Immediate perfusion of the tissue accomplished three objectives required in order to obtain viable hepatocytes: 1) removal of blood from the tissue before it clotted; 2) removal of calcium from the tissue which expedites dissociation; and 3) rapid cooling of the tissue to extend viability. The perfused tissue was transported quickly to the laboratory in ice-cold perfusion buffer.

Any unblanched tissue located around the cut surface of the lobe was trimmed away. The lobe was perfused as before with an additional 400 ml of perfusion buffer at room temperature. A 20 g portion of the liver was excised from the lobe with as much intact capsule as possible. Usually, greater success was achieved with the apical region of the caudate process because of a higher ratio of intact capsule to cut surface. The 20 g portion of liver then was placed in the perfusion apparatus.

The perfusion apparatus was composed of a 600 ml beaker that served as a reservoir and a wide stem glass funnel 100 mm in diameter containing a 150 ml sintered glass bottomed crucible placed on top of the beaker (reservoir). The crucible, which functioned as a support surface for the lobe, had several 1 to 2 mm diameter holes bored in it to facilitate drainage. The perfusion apparatus was placed in a 37^{o}C water bath. A two-channel perfusion pump (Masterflex, Barrington, IL) circulated the collagenase solution through the tissue via catheters sutured into exposed arteries or veins.

An alternate but less desirable procedure was to attach

20 Ga x 3.8 cm needles to the ends of the perfusion tubing and insert them into the liver tissue. This procedure periodically was used as a back up method to the aforementioned suture technique. When the needles were used, they had to be moved to different locations within the tissue at frequent intervals in order to achieve an even perfusion.

The perfusion flow-rate was approximately 15 ml/min from each outlet. The collagenase solution was kept at 38 to 39^{o}C to compensate for the temperature drop in the perfusion apparatus. Optimal cell disaggregation was achieved when the collagenase solution was not recirculated through the tissue and the tissue capsule remained intact. The collagenase perfusion was terminated after the tissue became swollen, took on a mushy appearance and the capsule became wrinkled with collagenase solution exuding from all intact surfaces. This generally took 30 to 45 minutes.

The perfused tissue then was placed in a 150 mm plastic petri dish containing a small amount of collagenase solution, and cut into 1 to 1.5 g pieces. A stainless steel comb was used to dislodge the cellular material from the capsule and connective tissue. Next, 2 to 3 volumes of ice-cold wash buffer were added to the dispersed tissue, the mixture of single cells and debris were filtered through 150 micron mesh nylon, and the remaining residue was discarded. Alternatively, a two-step filtration through a 250 micron followed by 150 micron mesh nylon was occasionally necessary to prevent clogging of the finer mesh nylon. The filtered suspension was then poured into

cold 15 ml plastic centrifuge tubes and centrifuged for 3 min at 50 x g with the supernatant being discarded. The cells were washed a total of three times with cold wash buffer and were resuspended by using gentle action with a wide bore Pasteur pipette.

After the final wash, cells were reconstituted to a thick suspension of greater that 1.0×10^7 cells/ml in the wash buffer and either used immediately or stored for short periods in an ice bath. The entire isolation procedure required 2 to 3 hours.

Cell Counting and Trypan Blue Dye Exclusion Viability Determination

A volume of 0.1 ml of the cell suspension was added to 0.9 ml of trypan solution and left standing for 5 min before the cells were counted in a hemocytometer. Both stained (non-viable) and unstained (viable) cells were counted and the viability percentage and number of viable cells per milliliter were calculated. The cell suspension was diluted to a working concentration of 5 to 10×10^6 viable cells/ml. Two ml of cells at this concentration and at 75 plus percent viability were equivalent to 10 to 20 mg/ml dry weight.

Gluconeogenesis

Two ml of lactate and control media (Krebs et al., 1976, 1979) were added to respective 30 ml culture flasks along with 2 ml of isolated hepatocytes. Incubation of media and cells was at $37^{\circ}C$ in a shaking water bath. Flasks were gassed with 95% O_2/5% CO_2 at the start of the incubations and every 30 min thereafter. Incubations were terminated after

various times by addition of 120 ul of concentrated (70%) perchloric acid followed by centrifugation for 15 min at 900 x g. The supernatant was used for the glucose assay, and the pellet was discarded. The glucose concentration was determined as described by Raabo and Terkildsen, (1960). Endogenous glucose production was the difference between controls incubated for the various times and a zero time. The zero time value was obtained by immediately stopping the reaction between media and isolated hepatocytes.

In experiments that compared isolated hepatocytes with liver slices, tissue was obtained from the caudate lobe of the same animals. The isolated hepatocytes were prepared as described. Slices were prepared by slicing on a Stadie-Riggs microtome (Thomas Scientific) which was calibrated to deliver slices 0.8mm thick. The sections were kept in ice-cold wash buffer until used.

Dry weights were obtained from the difference between 2 ml of a working concentration of isolated cells and 2 ml of wash buffer dried for 2 h at 100^{o}C. Viable dry weight is a term that describes the percentage dry weight resulting from viable hepatocytes as determined by trypan blue.

Palmitic Acid Oxidation

Liver slices (5 to 30 mg dry weight) and isolated hepatocytes (2 ml, 10 to 20 mg dry weight) were placed in 25 ml Erlenmeyer flasks containing 3 ml of oxidation media for the incubation of slices or 2 ml for incubation of isolated hepatocytes. Flasks were equipped with suspended center wells, gassed

for 15 s with 95% O_2/5% CO_2 and incubated for 60 min at 37°C. Reactions were terminated by addition of 3 ml of 1M perchloric acid. Methylbenzethonium hydroxide (0.3 ml) was added to the center wells and incubations were continued for an additional 60 min to collect liberated $^{14}CO_2$. Radioactivity in $^{14}CO_2$, and acid-soluble metabolites was determined by liquid scintillation counting (Nuclear Chicago, Model Mark 1) in ASC scintillant (Amersham). Quench corrections were calculated from a standard quench curve.

Acid soluble metabolites were identified by high performance liquid chromatography on Waters Associates equipment (Milford, MA) and consisted of a M45 solvent delivery system, a model 441 detector set at 214 nm and a C_{18} reverse phase Radial-Pak cartridge.

Oxygen Utilization

Oxygen utilization was determined by monitoring the disappearance of oxygen at 25°C from a suspension of isolated hepatocytes in oxygen containing media. An oxygen specific electrode (Zazar Research Laboratories DO-166 dissolved oxygen probe, Los Angeles, CA) was used to monitor oxygen. The oxygen probe was fitted through an aperture in the cap of a 30 ml plastic vial. The vial contained 30 ml of Hanks balanced salt solution oxygenated at 20 ppm, and approximately 2.0×10^7 viable cells. When the oxygen probe was in place, no air pockets were contained within the vial. The vial itself was sealed with layers of parafilm so that exchange of gases with the atmosphere was minimal. The oxygen probe and its interface unit were attached

to a pH meter (Fisher Accumet Model 525 Digital pH/ion) capable of reading millivolts. The mixture of isolated hepatocytes and oxygenated media was mixed gently with a micromagnetic stir bar and a Corning PC-353 magnetic stirrer. The speed of the micromagnetic bar was controlled by a variable autotransformer in line with the Corning magnetic stirrer.

Once an oxygen baseline was established, the recorder was turned on, and the slope of the oxygen disappearance was monitored. Controls consisted of cyanide-treated (nonviable) isolated hepatocytes analysed in the same manner. The oxygen probe was calibrated to atmospheric oxygen and to a 0 ppm oxygen standard prepared by dissolving 1.6 g of sodium bisulfite in double-distilled water.

Cytochromes P-450 and b_5

Cytochromes P-450 and b_5 were quantitated in microsomes prepared from intact liver and microsomes prepared from isolated hepatocytes. A portion of the caudate process was used for microsomal preparation. The remainder of the caudate lobe was processed as described to obtain isolated hepatocytes. The method used for the preparation of microsomes was described by Shull et al. (1982).

Microsomes were resuspended in 150 mM KCL to yield approximately 30 mg of protein/ml. Protein was determined colorimetrically (Gilford Stasar II Spectrophotometer model 136745) by the biuret method. BSA was used as the standard (Gornall et al., 1949).

Microsomal concentrations of cytochromes P-450 and b_5

were measured spectrophotometrically (Beckman Acta III dual-beam spectrophotometer) by the carbon monoxide difference spectral procedure. The microsomes were suspended in 100 mM tris buffer, pH 7.4, at a protein level of 1.0 mg/ml (Omura and Sato, 1964).

Epoxidation of Aldrin

Aldrin was dissolved in either DMSO or ethyl alcohol at 2 mg/ml and added to isolated hepatocytes in suspension culture. Either 10 ug, 20 ug or 50 ug of aldrin were added to culture flasks containing 2 ml of phosphate buffered saline and viable hepatocytes equivalent to 8 to 10 mg dry weight. The flasks were gassed with 95% 0_2/5% CO_2 and incubated at 37°C in a shaking water bath for either 15 or 30 min. Reactions were stopped by adding 10 ml of a 3:2 mixture of n-hexane and isopropyl alcohol. This solvent was also used to extract the aldrin and dieldrin (3X). The solvent was evaporated and analysed for aldrin and dieldrin with a Varian 2100 gas chromatograph (Palo Alto, CA) equipped with an electron capture detector. The column was glass, 1.83 m long, 2 mm ID, packed with 4% SE-30 on 100/200 Cromosorb W .

Statistical Analysis

Data were analyzed by regression analysis and analyses of variance on the Genstat V system at Michigan State University, East Lansing, Michigan.

A model describing the relationships among the variables affecting either cytochrome P-450 or cytochrome b_5 concentration

was used. It was assumed that the same variables affect both cytochrome concentrations. The model describing this relationship was: $Y_{ijkm} = M + T_i + M_j + T.M_{(ij)k} + e_{ijkm}$

where Y_{ijkm} is the P-450 or b_5 cytochrome concentration,

M is the mean of all responses,

T_i is the effect of the i-th tissue (i = 1 isolated cells or 2 intact tissue),

M_j is the effect of the j-th time (j = 1, ..., 6; representing six different times),

$T.M_{(ij)k}$ is the effect of the k-th interaction of tissue with time (k = 1,..., 6: representing six tissue time interactions),

e_{ijkm} is the residual error.

$T.M_{(ij)k}$ is the error term for T_i and M_j.

Factors T_i, M_j, and $T.M_{(ij)k}$ are fixed, whereas Y_{ijkm} and e_{ijkm} are random (Gill, 1978).

RESULTS

Viability and yield of hepatocytes isolated from each of 20 caudate processes are presented in Table 4.1 with an example of the ultrastructure of freshly isolated bovine hepatocytes shown in Figure 4.1.

Figure 4.2a shows total glucose production by isolated bovine hepatocytes over 2 h. This figure shows a slightly greater rate of increase of total glucose formed in hepatocytes incubated in the lactate substrate than in the control media. This can be attributed to the depletion of endogenous precursors for gluconeogenesis and glycolysis in hepatocytes incubated in the control media. Most of the glucose formed can be attributed to glucose production from endogenous precursors. The difference between the two traces (total glucose production in lactate-fortified cells and glucose production in control cells) represents glucose production attributed to gluconeogenesis from the nonendogenous substrate, primarily lactate. The rate of glucose production due to exogenous substrate is presented in Figure 4.2b. The rate of glucose production from exogenous sources decreased during the 2 hr incubation. However, the total amount of glucose produced over the same time increased.

The potential advantage of isolated hepatocytes over slice techniques is shown in Table 4.2. This table shows comparative data on gluconeogenesis for both isolated hepatocytes and liver slices. The liver for both methods was obtained from the same animal and analysed simultaneously. Isolated

Table 4.1.

Yield and Viability After Isolation Of Bovine Hepatocytes[a]

Trial #	% Viability[b]	Total Number of Viable Cells Obtained x 10^8
1	91.2	.12
2	68.0	2.09
3	81.0	2.64
4	88.2	5.56
5	86.6	.89
6	91.6	12.00
7	88.8	5.73
8	87.7	6.80
9	76.2	1.80
10	63.0	2.85
11	80.2	1.95
12	87.3	4.82
13	80.3	1.21
14	80.2	4.98
15	82.2	2.93
16	70.2	2.95
17	70.3	.77
18	73.2	2.24
19	87.0	1.29
20	92.0	.34
	81.3% mean viability	3.20 x 10^{8c} mean total viable cells

[a]Starting with liver tissue wet weights of 12 to 20 grams.

[b] Viability as determined by trypan blue dye exclusion.

[c] Corresponds to 3.94 x 10^8 total cells isolated (viable plus non-viable as determined by trypan blue) and 21.6 x 10^6 viable hepatocytes per gram of tissue (wet weight).

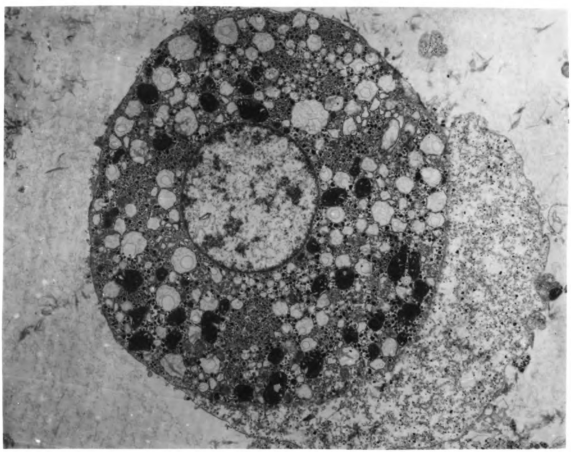


Figure 4.1. Isolated Bovine Hepatocyte

Figure 4.2a. Total Glucose Production

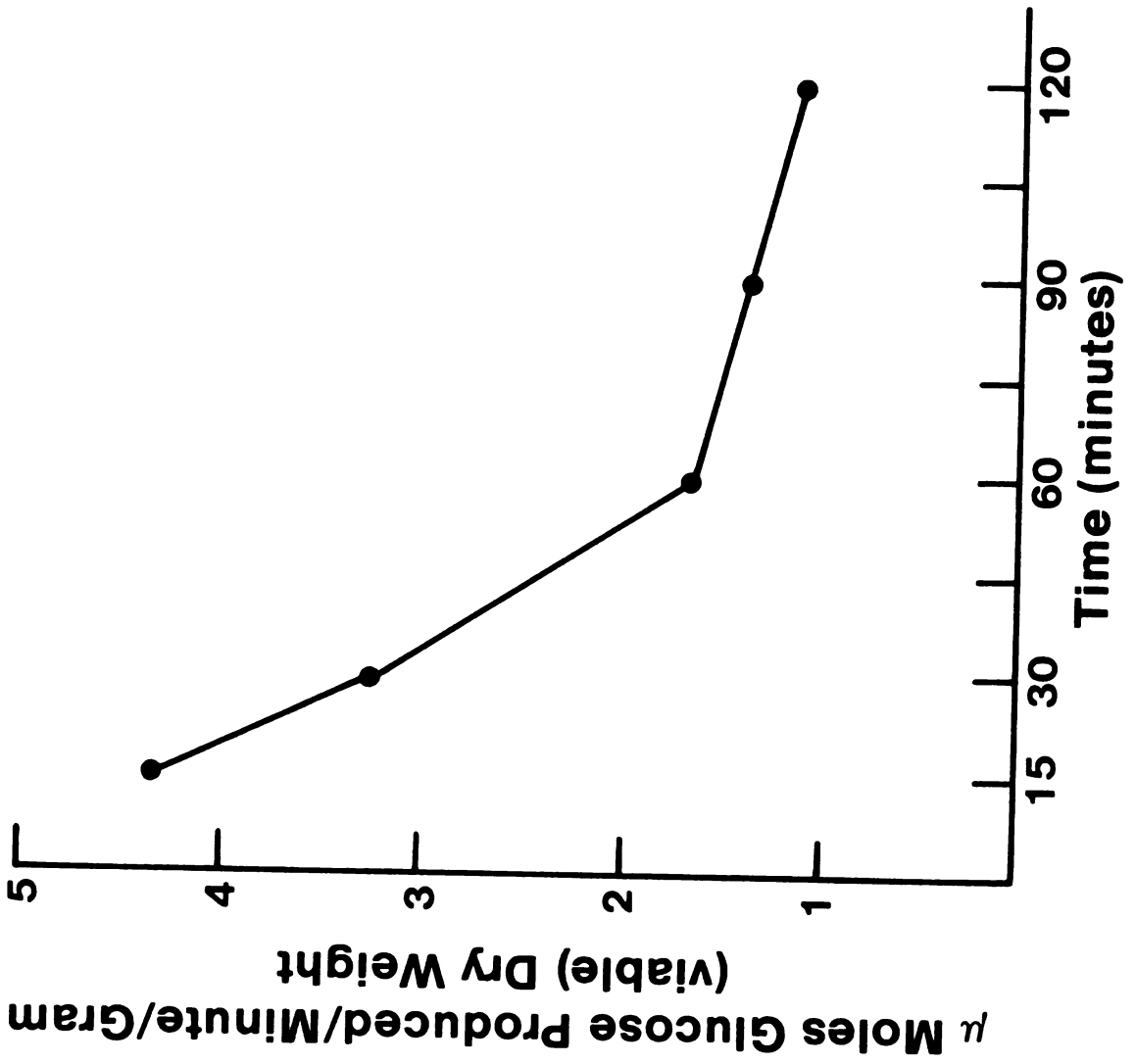

μ Moles Glucose Produced/Minute/Gram
(viable) Dry Weight
5
4
3
2
1
15
30
60
90
120
Time (minutes)

Figure 4.2b. Rate of Glucose Production from Exogenous Substrate in Bovine Isolated Hepatocytes.

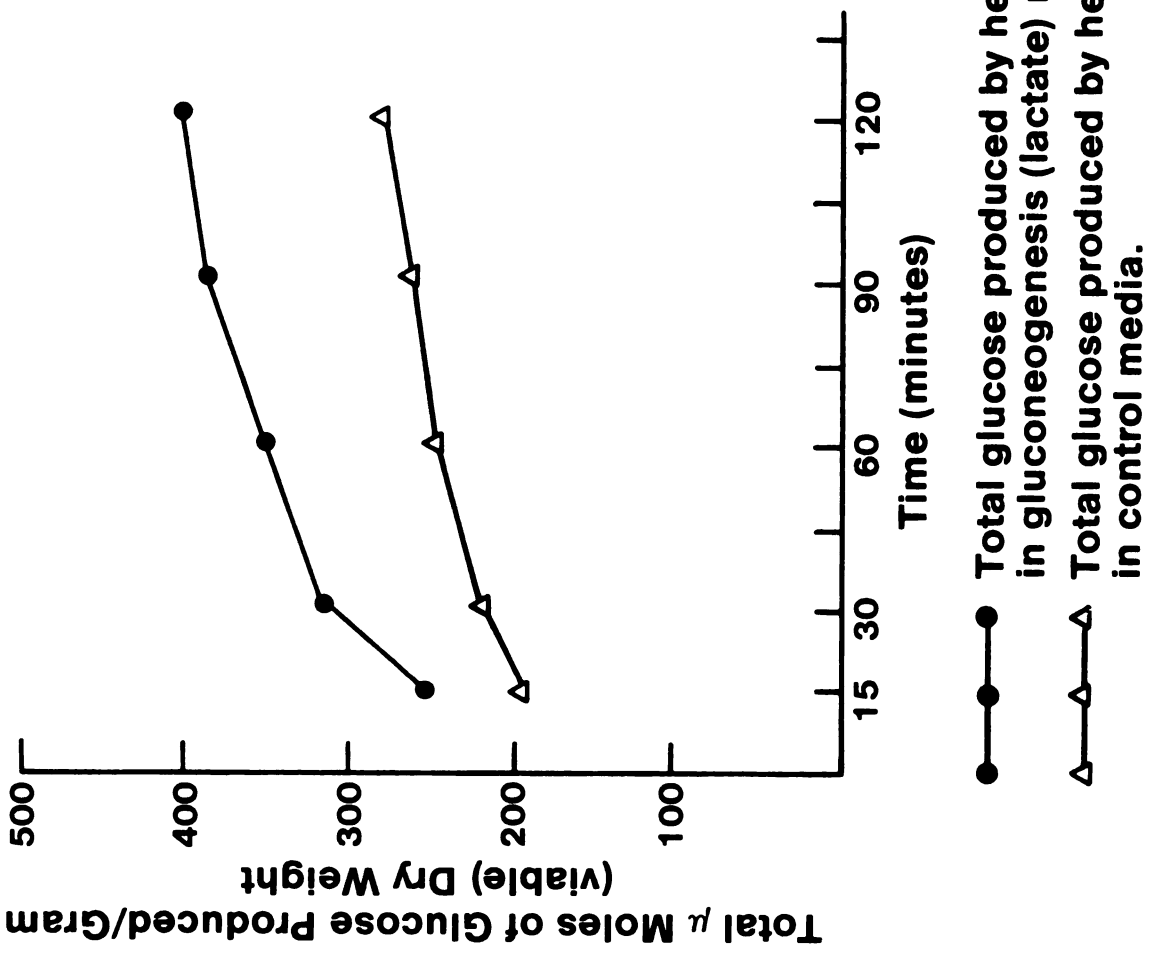

Total μ Moles of Glucose Produced/Gram (viable) Dry Weight
500
400
300
200
100
15
30
60
90
120
Time (minutes)
Total glucose produced by hepatocytes in gluconeogenesis (lactate) media.
Total glucose produced by hepatocytes in control media.

TABLE 4.2.

Comparison of Gluconeogenesis Between Bovine Isolated Hepatocytes and Liver Tissue Slices[a]

Animal	Substrate	Isolated Cells	Tissue Slices
	Lactate[b]	1.47 ± .12[e]	None detectable
		(1.68) ± .14[f]	
1	Propionate[c]	1.01 ± .03	None detectable
		(1.16) ± .04	
_____	None[d]	3.81[g]	1.43
	Lactate	.88 ± .07	.12 ± .93
		(1.15) ± .09	
2	Propionate	.13 ± .07	None detectable
		(.18) ± .10	
_____	None	5.26	2.90

[a]Isolated hepatocytes and liver slices were from the same animal and were analyzed at the same time.

[b]Earle's balanced salt solution without glucose and phosphate: with a final concentration of 10 mM lactic acid, 1mM lysine, 1mM pyruvate, 10mM sodium acetate, 0.2mM dibutyryl cAMP, pH 7.45 saturated with 95% O_2/5% CO_2 and run in triplicate.

[c]Same as b except propionate (10mM) is substituted for lactate.

[d]Earle's balanced salt solution without glucose and phosphate: with a final concentration of 10mM sodium acetate, 0.1mM dibutyryl cAMP, pH 7.45 and saturated with 95% O_2/5% CO_2.

[e]umoles of glucose produced/minute/gram of liver (dry weight) at 37°C at 60 minutes ±SE.

[f]umoles of glucose produced/minute/gram of liver ("viable" dry weight) at 37°C at 60 minutes ±SE.

[g]Glucose produced from endogenous sources in umoles produced/minute/gram of liver (dry weight) at 37°C at 60 minutes.

hepatocytes showed higher gluconeogenesis rates when calculated on a dry weight basis than did tissue slices. It is also possible to calculate results as per gram "viable" dry weight when isolated hepatocyes are used. This calculation is justified because hepatocyte preparations showing virtually 100% nonviable cells by trypan blue dye exclusion will not exhibit gluconeogenesis.

Further comparisons between isolated hepatocytes and liver slices were made on their ability to oxidize palmitate (Table 4.3). The rate of oxidation of 1-^{14}C palmitate to $^{14}CO_2$ and ^{14}C- acid soluble metabolites by isolated hepatocytes was four to five times higher than in liver slices. This comparison is on a pmoles of 1-^{14}C palmitate oxidized to product/minute per mg tissue (dry weight) basis. The majority (>70%) of radioactivity in the acid-soluble fraction was B-hydroxybutyrate and acetoacetate as determined by high performance liquid chromatography. A comparison of isolated cells and tissue slices on a dry weight basis is not entirely accurate because of the connective tissue in liver slices which contributes to the dry weight of the slice. However, it is believed that the difference caused by this discrepancy is small in comparison to the differences between rates of metabolism.

This rate of oxygen uptake by viable isolated hepatocytes is presented in Table 4.4 and indicates that the viable hepatocytes consumed oxygen almost three times as fast as nonviable hepatocytes. Oxygen utilization in the nonviable hepatocytes was probably due to non-biologic utilization of oxygen. The addition

TABLE 4.3

Comparison of Palmitic Acid Oxidation by Bovine Liver Slices and Isolated Bovine Hepatocytes

1-^{14}C-Palmitate oxidation to	Slices		Cells
	Wet Weight	Dry Weight	Dry Weight
$^{14}CO_2$	0.36 ± .04[a]	3.16 ± .50	14.90 ± 2.34[b]
^{14}C-Acid Soluble Metabolites	1.16 ± .16	10.77 ±2.03	47.99 ± 8.18[b]

[a] Mean ±SE in pmoles 1-^{14}C palmitate oxidized to product/min/mg tissue of quadruplicate incubations from six different tissue preparations.

[b] Significantly different from slices, ($P < .001$).

TABLE 4.4

Oxygen Utilization as an Indicator of Isolated Hepatocyte Viability.

Isolated Hepatocytes	Oxygen Utilization	Viable/Nonviable Ratio
Viable[a] (5)[b]	0.48 ± .08[c]	2.82
Nonviable[d] (3)	0.17 ± .01	

[a]Viability as determined by trypan blue dye exclusion (0.4% for 5 minutes). Viable cells had a mean of 75.2% viability which represents 21.7 million variable cells per assay.

[b]Number of different isolated hepatocyte preparations tested.

[c]Mean slope ±SE of oxygen disappearance. The slope for the viable hepatocytes represents an oxygen disappearance rate of 9.6 ppm 0_2 per hour compared to a rate of 3.4 ppm 0_2 per hour for the nonviable cells.

[d]Virtually 100% as determined by trypan blue dye exclusion after treatment with potassium cyanide.

of 1mM succinate, which stimulates respiration in cells with damaged plasma membranes (Baur et al., 1975) did not cause an increase of oxygen uptake.

Levels (nmoles) of cytochromes P-450 and b_5 per mg protein, two key cytochromes involved in xenobiotic metabolism, were compared between isolated hepatocytes and microsomes. Both isolated hepatocytes and microsomes were prepared from hepatic tissue taken from the same animal. Results of analyses of liver tissue for microsomal cytochromes were compared with those obtained using isolated hepatocytes kept for up to 5.5 h. This interval provides adequate time for isolation of hepatocytes (i.e., 2h) and a metabolism study (i.e., 3.5 h). Results of statistical analysis as shown in Table 4.5 indicated that both cytochrome P-450 and b_5 in isolated hepatocytes were not significantly different from initial cytochrome concentrations for up to 5.5 h.

The activity of mixed function oxidases (MFO), of which cytochrome P-450 is one, in isolated hepatocytes is illustrated in Table 4.6. As a result of the MFO mediated reactions, oxygen is added to the test xenobiotic aldrin to form the stable epoxide dieldrin.

Table 4.5

Comparison of Cytochromes P-450 and b_5 Levels Between Fresh Bovine Liver Tissue and Isolated Hepatocytes[a]

Cytochrome	P-450		b_5		Time (min)
	Cells	Tissue	Cells	Tissue	
Trial 1	.220[b]	.523	.170[b]	.177	195[c]
2	.541	.476	.395	.420	195
3	.429	.644	.292	.439	195
4	.467	.341	.278	.269	210
5	.418	.418	.273	.216	225
6	.352	.703	.216	.246	240
7	.390	.819	.336	.319	255
8	.363	.620	.322	.296	330
$\bar{x}$	.398[d]	.568	.285[e]	.298	
SE	±.033	±.056	±.025	±.033	

[a]Tissue and isolated hepatocytes analysed for cytochromes P-450 and b_5 were obtained from the same animal. Tissue was processed for cytochromes immediately upon procurement.

[b]nmoles cytochrome/mg protein.

[c] Time in minutes after tissue that the isolated hepatocytes were processed for cytochromes determinations.

[d]Not significantly different from tissue ($P > .05$).

[e]Not significantly different from tissue ($P > .10$).

TABLE 4.6

Epoxidation of Aldrin By Bovine Isolated Hepatocytes[a]

Incubation Time	Aldrin added	Delivery solvent[b]	Dieldrin found	Aldrin to dieldrin[c]	Conversion rate
Min.	ug	--	ug	% conver.	ug/min/g (viable) dry weight
15	50	ETOH	0.84	2.47	6.4
30	50	ETOH	1.2	3.24	4.6
30	50	DMSO	0.68	1.67	2.6
15	20	ETOH	0.68	5.01	5.2
30	20	ETOH	0.65	5.10	2.5
15	10	ETOH	0.57	6.35	4.4
30	10	ETOH	0.40	4.88	1.5
30	10	DMSO	0.60	5.77	2.3

[a] 5.6 to 10.4×10^6 viable hepatocytes per culture.

[b] Ethyl alcohol (ETOH) or dimethyl sulfoxide (DMSO).

[c] No conversion occurred in cultures containing an equivalent weight of intact nonviable isolated hepatocytes.

DISCUSSION AND CONCLUSIONS

As shown in Figure 4.1, the isolated hepatocytes were in a morphologically intact state having normal membranes and organelles. However, swelling of the endoplasmic reticulum is evident. Whether these swollen endoplasmic reticulum contain lipid or whether they would return to normal with time is not known.

High yields of cells are desirable, however, yield should not be used solely to judge the quality of hepatocyte preparations. Biochemical and physiological function are much more useful to ascertain hepatocyte quality since cell yield depends on factors which have no major influence on the viability of isolated cells. Some factors which affect yield include the number of washing (purification) steps and the centrifugation speed. Seglen (1973b) reported an increase from 30 to 90% in liver weight recovered as suspended cells by decreasing the centrifugation force and number of washing steps. Accompanying this decrease in cell yield was an increase in the purity of parenchymal cells from 80% to greater than 98%.

Trypan blue exclusion is not a reliable indicator of cell function (Krebs et al., 1979). Vital stains indicate only irreversible damage to cells. Also, staining can be affected by environmental factors such as pH (Baur et al., 1975). It has been the author's experience that trypan blue accurately identifies cells that are nonviable, but it sometimes gives false negative (nonstaining) results as compared with other viability measurements such as gluconeogenesis or oxygen

utilization. Because of this, it is suggested that several cellular function tests be used to adequately judge hepatocyte viability.

Perhaps the most sensitive indicator of cellular viability is gluconeogenesis from precursors such as lactate (Krebs et al., 1979). To synthesize glucose from lactate, a complex anabolic process, cells must possess intact cellular components and metabolic processes such as mitochondrial and plasma membranes, numerous enzyme functions, adequate energy supply and regulatory mechanisms (Hemes et al., 1966; Krebs et al., 1979).

The rates of gluconeogenesis achieved (Figure 4.2 and Table 4.2) are consistent with those for ovine isolated hepatocytes fortified with 10mM lactate, (Ash and Pogson, 1977) a gluconeogenesis rate of 1.41 umoles of glucose formed/minute per g of liver dry weight. Also in ovine isolated hepatocytes,and fortified with 10mM lactate, Clark et al. (1976) reported gluconeogenesis rates ranging from 1.6 to 2.2 umoles/minute per g liver (dry weight).

Clark et al.,(1976) also evaluated a whole liver perfusion technique to measure glucose formation from 10mM lactate. With this procedure, Clark obtained gluconeogenesis rates ranging from 1.1 to 2.5 umoles/minute per g liver (dry weight). Cook (1966) reported that the total body glucose turnover rate in a cow was 0.873 grams/min. This figure can be used to calculate umoles of glucose formed/minute per g of liver (dry weight) assuming a cow's liver weight at 0.75% to 1% of body

weight (Cook, 1966; Kinzell et al., 1981) and a wet weight to dry weight conversion factor of 3.7 (determined from the experiments). Thus, a range of gluconeogenesis rates of between 4.3 and 5.7 umoles glucose formed/minute per g of liver (dry weight) is derived. This range of calculated gluconeogenesis rates is consistent with the data, especially when glucose production from both exogenous and endogenous sources are added together. Taken together, trypan blue dye exclusion, gluconeogenesis, ketogenesis and oxygen uptake results provide excellent evidence that the isolated hepatocytes are viable.

The main purpose for establishing a method for isolation of viable and functional hepatocytes from bovine liver was to provide a system for studying xenobiotic metabolism in vitro. Because of significant differences among species in the metabolism of xenobiotics (Kulkarni and Hodgson, 1980), it is best to employ the species of research interest in order to obtain relevant data. Although sometimes preferred, xenobiotic studies carried out in a live animal such as the cow are impractical. Isolated rat hepatocytes have been used in xenobiotic metabolism studies (Dougherty et al., 1980; Salocks et al,1981) and should become even more practical for studies in nonlaboratory species such as cattle.

Since statistically significant changes in cytochrome levels were not detected, neither cytochrome, P-450 or b_5, should be rate limiting in short studies of xenobiotic metabolism. There were, however, numerical reduction of P-450 in

some cell preparations. This phenomenon was noted in rats (Michalopoulos et al., 1979) in which only 10% of the original cytochrome P-450 remained after four days of culture. In humans, 50% remained after four days (Strom et al., 1982). These findings illustrate the importance of short xenobiotic metabolism studies until P-450 either can be induced in culture or maintained longer. Research indicates that maintenance of P-450 may soon be possible. Nutritional requirements (Paine and Hockin, 1980) are being defined so P-450 can be maintained in vitro. Additives such as ascorbic acid (Bissel and Guzelian, 1979), 5-aminolevulinic acid, and various hormones (Michalopoulos et al., 1976; Decad et al., 1977) also seem to be beneficial.

Losses of cytochrome P-450 during the postisolation period is the major problem in using isolated hepatocytes for metabolism studies. Most of the variance in cytochrome levels accounted for by statistical analysis was attributed to the particular tissue used for analysis (28.2%) and was not due to the amount of time elapsed after removal of the liver (14.5%). This indicates that the majority of the cytochrome lost was due to something associated with the tissue. It is suspected that cytochrome P-450 may be lost from nonviable and damaged cells and is being removed during the washing procedure.

The aldrin epoxidation model illustrates how intact isolated hepatocytes can be used to activate chemicals in vitro. This activation system may more accurately model chemical activation in mammals than do microsomal systems.

Studies using isolated hepatocytes are rapidly emerging as an effective approach for evaluating biochemical pathways. This chapter provides a description of the means in which viable isolated hepatocytes can be easily prepared from large animals. Bovine isolated hepatocytes should be useful to the bovine nutritionist who is studying metabolism kinetics, as well as to the toxicologist.

SUMMARY

This research supports the following conclusions: First, exposure to environmentally relevant levels of pentachlorophenol (less than 1 mg/kg body wt/day) does not adversely affect the immune system of either lactating adults or bull calves. This statement does not extend to all situations of pentachlorophenol exposure. Exposure to pentachlorophenol may cause altered host resistance when acting concomitantly with another stress factor such as inadequate nutrition, poor ventilation, or simultaneous exposure to other toxic substances.

Second, at high levels of pentachlorophenol ingestion (greater than 1 to 2 mg/kg body weight per day in calves) the immune system is adversely affected. Prolonged exposure to pentachlorophenol at these levels causes thymic atrophy, and a decreased lymphocyte blastogenesis in thymocytes. Clinicopathological changes include elevation of serum gamma-glutamyl transferase, a result of microsomal induction by the dioxins and other contaminants in pentachlorophenol, and reductions in serum total protein and albumin. The reduced total protein and albumin is probably a secondary effect associated with protein deficiency caused by reduced feed intake and gastointestinal irritation. These are both attributable to pentachlorophenol ingestion. High levels of pentachlorophenol also inhibit shape

change in bovine neutrophils *in vitro*. However, no effect was seen in any neutrophil parameter studied *in vivo* and neutrophil chemotaxis was not studied.

Third, it is apparent that adverse effects of pentachlorophenol exposure were caused more by the toxic impurities than the pentachlorophenol itself. Thus, wood treated with the pure pentachlorophenol would be the safest to use, if contact with cattle is expected.

Last, viable isolated hepatocytes can be used as a means to activate xenobiotics *in vitro*. An isolated hepatocyte system takes structure and kinetics into account in metabolism and should prove useful in the study of xenobiotics.

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