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PLUTONIUM-LYMPH RELATIONSHIP

By

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MASTER

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A Study of the Translocation of Plutonium and
Americium From Puncture Wounds

Directed by Principal Investigator

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ABSTRACT OF DISSERTATION

THE PLUTONIUM-LYMPH RELATIONSHIP

Uptake and transport mechanisms of soluble ($\text{Pu}(\text{NO}_3)_4$) and insoluble (polydispersed PuO_2) plutonium were studied in afferent and efferent lymph from the popliteal lymph nodes of dogs after simulated wound exposures. Normal values for constituents of canine lymph were determined. The validity of using electrophoretic techniques in studying the Pu-lymph relationship was evaluated. Lymph transport of monodispersed PuO_2 particles (0.16μ , 0.54μ , and 1.05μ) was compared.

The normal lymph cell count was 220 cells/mm^3 in afferent lymph and 7398 cells/mm^3 in efferent lymph. Protein concentration in afferent lymph was $13.81 \pm 4.02 \text{ mg/ml}$, while efferent lymph contained $21.43 \pm 2.80 \text{ mg/ml}$.

Discontinuous and isoelectric focusing gel electrophoretic systems were useful in determining which proteins bind Pu, but were inaccurate in determining percentages of Pu binding to specific proteins.

Plutonium nitrate concentration was higher in the acellular fraction of both afferent and efferent lymph, while a greater concentration of PuO_2 was found in the cellular fraction.

Particle size was not found to be a factor in the total movement (acellular and cellular lymph fractions) of Pu in afferent and efferent lymph. Regardless of particle size, more Pu moved in the cellular fraction of both afferent and efferent lymph than in the acellular fraction.

Discontinuous gel, isoelectric focusing gel, and cellulose acetate strip electrophoresis indicated that transferrin and albumin were the two principal proteins transporting Pu in the acellular fraction of both afferent and efferent lymph.

Immunoelectrophoresis proved to be an inaccurate technique to study the protein-bound plutonium.

Fission track analysis indicated that dissolution of Pu particles was taking place at the wound site. Plutonium oxide particles at the implant site were larger than in afferent lymph and larger in afferent lymph than efferent lymph.

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CHAPTER I

INTRODUCTION

Plutonium research is increasing in importance due to its proposed use in breeder reactors as the energy source of the future (Bair and Thompson, 1974). This will lead to a concomitant increase in handling of plutonium by man and thus increase the risk of accidental human exposures. Many of the human plutonium exposures that have already occurred involve wounds (Bistline, 1973). The kinetics of Pu movement from a wound site has been studied here at Colorado State University but only recently has a mechanistic approach to the plutonium-lymph relationship been attempted. Gomez (1973) studied the movement of polydispersed PuO_2 in afferent lymph and reported that beta-globulins were the most important protein group in transporting plutonium in lymph. Bistline (1973) indicated the need for studies involving monodispersed PuO_2 particles because of the large difference that has been observed between the movement of air-oxidized polydispersed PuO_2 particles (geometric mean diameter of 7.0μ) and high-fired polydispersed PuO_2 particles (geometric mean diameter of 0.7μ).

This study was initiated to answer the following questions:

1. How is Pu being transported in the lymph? How is the

protein molecule transferrin actually involved in this movement?

2. What differences occur in the binding of plutonium in the afferent and efferent lymph channels to and from the popliteal lymph node?

3. How does the binding of soluble and insoluble chemical forms of plutonium in the lymph compare?

4. What effect does particle size have on the movement of Pu from a wound site? How do particles found in afferent and efferent lymph differ in size?

CHAPTER II

REVIEW OF LITERATURE

I. History of Plutonium

Plutonium has steadily ascended the ladder of importance in the nuclear industry since its discovery in February, 1941 by Dr. Glenn T. Seaborg and associates (Seaborg, 1963). Early uses of plutonium were primarily in the nuclear defense industry but it will figure heavily in the production of power in breeder reactors in this country during the next several decades. It is predicted that plutonium fuel will produce 50% of our power before the end of the century (Seaborg, 1972). Therefore, the prospects for accidental exposure of the human populace, both working and non-working, must increase perforce with this increased utilization of plutonium as an energy source.

At present there are only a few individuals known to have plutonium body burdens in excess of the ICRP limits of $.04 \mu \text{ Ci}$; however, over a thousand persons are known to have detectable plutonium body burden levels (Bistline, 1973). No serious work injury attributable to plutonium deposition has been reported in the literature (Norwood, 1972).

II. Chemistry of Plutonium

The 239-isotope of plutonium is a major concern because of its use as a fissionable fuel in power breeder type reactors and in explosive nuclear devices (Bair and Thompson, 1974). The various radionuclides commonly found in reactor-produced plutonium were described by Johnson (1969).

The chemistry of the element plutonium is complicated. This is partly due to the five oxidation states that it can assume; +3, +4, +5, +6, and +7 (Taylor, 1973). The intensity of the oxidizing or reducing conditions on a solution affects the relative proportion of the various species in solution (Polzer, 1971). In a physiological situation, the +4 state is usually the most abundant, but percentages of the first four oxidation states can exist. Aqueous or dissolved Pu ions exist in a hydrated form and should be written as $\text{Pu}(\text{OH})_4$. This often leads Pu to polymerize and exist in a polymeric (particle) state. Although the polymerization rate increases with increasing pH, it can be depressed by large molar excesses of ions such as citrate (Lindenbaum and Westfall, 1965). This becomes especially important when comparing the effects of plutonium implanted subcutaneously with intravenously injected plutonium.

Industrial chemical forms of plutonium include PuO_2 (high-fired and air-oxidized), $\text{Pu}(\text{NO}_3)_4$, PuF_4 , and Pu_CI_3 . The plutonium oxide and plutonium fluoride chemical forms are considered to be

"insoluble", while plutonium nitrate and plutonium chloride are considered soluble forms (Cleveland, 1967). Plutonium oxide is considered to be the most important chemical form because of its usefulness as a nuclear fuel (Bair and Thompson, 1974).

Particle size is another important parameter to consider when discussing the chemistry of plutonium, especially PuO_2 . The ability of a particle to undergo dissolution or phagocytosis by a macrophage may to a large extent depend on its mean diameter (Dagle, 1973). The temporal relationship that exists between a particle's entrance into the body through a wound and its removal from the site via phagocytosis or dissolution may have important therapeutic implications.

III. Characteristics of Lymph

A review of the anatomy of the lymphatic system in the dog can be found in Miller et al. (1964). There are large differences in the characteristics of lymph depending on its location in the body (Yoeffy and Courtice, 1970).

Peripheral lymph, which has not yet passed through a lymph node, can be termed afferent lymph, and the relatively few cells which it contains are derived from the tissue in which its lymphatic capillaries originate (Yoeffy and Drinker, 1939).

Lymph which has passed through a lymph node can be termed efferent lymph. Cells in efferent lymph can originate from afferent lymphatic vessels reaching the node, from cells in the blood stream

which enter the node by passing through the walls of blood vessels, and from cells formed in the lymph node (Yoeffy, 1967).

The popliteal lymph node in most animals is oligosynthetic with minimal cell production (Mitchell et al., 1963).

Hayes and Field (1931) found 240 cells/mm³ in afferent lymph in the front limb of the active dog. Afferent lymph in sixteen dogs, studied by Yoeffy and Drinker (1939), contained 0 to 800 cells/mm³ with the highest percentage being lymphocytes. There is a small, but fairly consistent stream of macrophages to the regional lymph node in peripheral lymph (Yoeffy and Courtice, 1970). In 59 humans, Engeset et al. (1973) found an average concentration of 162 cells/mm³ in afferent lymph in the leg.

Smith et al. (1970) studied cell populations of afferent and efferent lymph vessels connected to the popliteal lymph node in sheep. For afferent lymph he found 200-700 cells/mm³ with 80-90% lymphocytes and 5-15% macrophages. In efferent lymph there were 3000-10000 cells/mm³ with 95-100% lymphocytes. Hall and Morris (1962) studied efferent cells from the popliteal lymph node in sheep and found 500-3500 cells/mm³ during the first four hours of cannulation. He also found that cell types changed, moving toward the more immature forms when stimulated by subcutaneous injections of human serum globulins.

The flow of lymph was seen to vary with the conditions under which it was collected. Smith et al. (1970) showed that flow in the peripheral lymphatics of sheep varied from 1.0 to 9.0 ml/hr.

The physiological role of the lymph system is the maintenance of homeostasis in the body as necessitated by capillary leakage, excess interstitial fluid or tissue damage.

The proteins in lymph are usually derived from surrounding circulating plasma. Protein content of a particular lymphatic is dependent on the capillaries which, in turn, depend on the fine structure of their walls and the hemodynamics of the particular region (Courtice, 1967). The lymph draining the limb is derived mainly from the vessels that normally course the skin and muscle.

Lymph contains essentially the same proteins as plasma but with variation in both concentration and distribution (Szabo et al., 1963). Winsey (1972), using starch gel electrophoresis, showed that in control afferent lymph 19 separate bands of protein can be demonstrated; however, their concentrations were lower than in plasma, especially for the proteins of higher molecular weight. Dive et al. (1971) again showed that protein concentrations in liver lymph were lower than in serum in dogs and that concentrations of particular proteins varied according to their molecular weight. Wasserman and Mayerson (1952) concluded, from their investigations in dogs, that

albumin leaks out of the capillaries 1.6 times faster than globulins and that they are picked up by lymph.

Drinker et al. (1934) showed that the popliteal lymph nodes of dogs have high filtering capacity. The question of mechanical versus biological removal has been discussed (Yoeffy, 1970; Dagle, 1973). It seems that flow through the lymph node dictates the ability of the lymph node to filter certain agents. Fluids and autogenous plasma passed through the lymph-venous communications in the popliteal lymph node of the dog (Pressman et al., 1962). Mayerson et al. (1962) showed that the popliteal lymph node of the dog did not trap molecules like dextran, molecular weight 400,000, and that very small molecules could pass into the plasma while transversing the lymph node. On the other hand, colloidal carbon is phagocytosed by macrophages in the lymph node (Napajoonsri and Simon, 1971). Perfusion of the popliteal lymph nodes in dogs with acacia-graphite suspensions resulted in deposition beyond the popliteal node despite the filtering mechanism (Drinker et al., 1934). Dagle (1973) has demonstrated the ability of macrophages to phagocytose Pu particles in the popliteal nodes of the dog.

IV. Plutonium and Lymph

Plutonium usually reaches the lymph system by inhalation or by translocation from a wound site. The plutonium deposits in the regional lymph nodes that drain the contaminated area.

Park et al. (1962) showed that inhaled Pu in dogs will translocate to the tracheobronchial lymph nodes. Cable et al. (1962) demonstrated Pu in prefemoral lymph nodes of a pig injected intradermally. Plutonium injected subcutaneously in the front limb of dogs deposited in the superficial cervical lymph node (Johnson, 1969). Dagle (1973) saw plutonium particles deposited in the popliteal lymph node of dogs injected subcutaneously with plutonium in the hindlimbs.

The lymph nodes have been used as a monitor of plutonium movement from a wound site (Johnson, 1969); Howells et al., 1972; Bistline, 1973; Dagle, 1973). It has been demonstrated that plutonium movement from a wound site to a lymph node is affected by chemical form, particle size, depth of implant, position of introduction, and individual biological variation (Bistline, 1973). Plutonium moves from a wound site to a lymph node via lymph and can be divided into cellular and acellular fractions (Gomez, 1973).

In the acellular fraction, the plutonium associates with specific proteins and is carried along in the flow. In serum, the highest concentration of plutonium molecules has been seen with transferrin (Stover et al., 1968). Gomez (1973) indicated that transferrin also carried plutonium in afferent lymph.

The cells of the cellular fraction phagocytize the Pu particle and carry it to the lymph node. Sanders (1972) and Sanders and Jackson (1972) have described the phagocytosis of PuO_2 particles by

macrophages. Plutonium particles have been localized in macrophages within the architecture of the popliteal lymph node (Dagle, 1973).

The importance of the lymph system in the movement of plutonium, which has come to light only recently, has necessitated the reassessment of therapeutic approaches to Pu wound contamination management.

V. Electrophoresis of Proteins

Many different methods have been employed in the separation and characterization of proteins. The method of choice is often dictated by the problem to be solved.

Electrophoresis is the transfer of charged elements from one location to another through mediation of an electric current. Electrophoretic analysis of protein mixtures by the moving boundary method was first adapted by Tiselius (Tiselius, 1937). Many advances in the applications of the technique and in the separating mediums employed have occurred since then (Bier, 1967).

A. Discontinuous Gel Electrophoresis

Polyacrylamide gel was first introduced as a supporting medium in zone electrophoresis in 1959 and led to the development of analytical and preparative techniques with great resolving power (Raymond and Weintraub, 1959). Since then polyacrylamide gel

electrophoresis has been utilized by a great many researchers to achieve the successful fractionation of proteins (Fredrick, 1964).

A molecular sieving effect in combination with charge separation results in the formation of fine, well-delineated protein bands. The ability of molecules to pass through a sieve depends on the size and shape of the holes in the gel, the size and shape of the molecules being sieved and interactions such as adsorption or ion exchange which may occur between the molecules and the matrix of the gel. Gel hole size or porosity of a polyacrylamide gel is dependent upon the amount of cross-linking within the gel which is directly related to the concentration of polyacrylamide versus the concentration of the cross-linking reagent (methylene-bis-acrylamide) (Gordon, 1972).

Often the best resolution is obtained with a discontinuous pH electrophoresis (disc electrophoresis) system. This system uses two different gel types and three different buffers.

An upper gel is used to stack or concentrate the protein and then the lower or resolving gel separates the protein mixture into well defined bands. Differences in the porosity of the gel layers and in the buffer types result in the improved resolution.

Estimation of radioactivity associated with a particular protein band has been achieved by slicing the gel into sections and then counting the radioactivity present with the proper detection equipment (Peacock and Dingman, 1968; Loening, 1967).

Polyacrylamide gel electrophoresis has been employed by two different researchers in correlating the location of a protein band (transferrin) with increased Pu content. Bruenger et al. (1969) used this method to show that Pu was associated with the plasma transferrin bands in low concentrations. Gomez (1973) reported a high percentage of Pu in the beta-globulin fraction using the same procedure.

B. Isoelectric Focusing Gel Electrophoresis

Isoelectric focusing electrophoresis has been used in both research and industry to separate all kinds of proteins according to their isoelectric point, pI, which is the pH value at which the dissolved protein has a net charge of zero (Catsimpoolas, 1973).

The charge that a protein carries is dependent on the solution in which it is dissolved. For example, when a specific protein is dissolved in a solution with a pH higher than the protein's pI value, the protein will lose protons and become negatively charged.

When the protein is added to a pH-gradient that has a pH range that includes the pI of the particular protein, the charges on the protein will depend on the pI of that protein and the particular pH at which it is located. If a voltage is then applied across this system, the protein will migrate toward the pH value where it is isoelectric, that is, has a net charge of zero. The protein is thus focused at the point where the pH is equal to the pI (Haglund, 1970).

Buffers or ampholytes are used to form the pH gradient in isoelectric focusing. They are synthetic polymers whose isoelectric points are carefully controlled and can be purchased with pH ranges between 3 and 11.

C. Cellulose Acetate

Electrophoresis, using cellulose acetate as the support medium, has been defined as a type of zone electrophoresis. In this method the sample is applied to a relatively inert support material where the charged particles (proteins) remain as discrete zones, thereby allowing chemical or physical characterization. As with other types of electrophoresis, zonation is dependent upon electric field strength, the characteristics of the protein (size, shape, net charge, degree of ionization), and the nature of the conducting fluid (Gelman, 1970).

Cellulose acetate has been utilized by researchers in England to separate and characterize the plutonium-protein complex (Turner and Taylor, 1968a). Using a $\text{Pu}(\text{NO}_3)_4$ solution incubated with horse serum it was shown that 70-80% of the plutonium came out with the β_1 -globulin fraction which contains transferrin. To further identify plutonium-protein relationships, Turner and Taylor performed in vivo experiments with rats, injecting different plutonium solutions and then performing cellulose acetate electrophoresis to identify plutonium associated with a particular protein fraction-again β_1 -globulin. This

was done with Pu citrate and $\text{Pu}(\text{NO}_3)_4$ (Turner and Taylor, 1968a). Thus, cellulose acetate has already been successfully employed as a separating medium for partial delineation of the plutonium-protein relationship.

D. Immuno-electrophoresis

Immuno-electrophoresis is a combination of electrophoresis and double diffusion systems. Wells are cut in agar-covered slides and the wells are filled with an antigen. When an electric current is applied, the antigen is carried some distance in the agar by the current but at the same time diffuses in a radial fashion. After the current is removed, a trough is cut into the agar parallel to the well and filled with antibody. Antibody diffuses from the trough and reacts with the antigen to form an arc-precipitate (Arquembourg et al., 1970).

Immuno-electrophoresis is often performed on serum to detect abnormal precipitate areas which may indicate qualitative or quantitative changes in the serum proteins which are otherwise difficult to detect.

Lymph has been studied using immuno-electrophoresis by several investigators (Szabo et al., 1963; Perlmann et al., 1943; Roberts and Courtice, 1969). The immuno-electrophoretograms of lymph and serum were reported to be essentially similar.

Demonstration of the capacity of certain proteins to bind small molecules may be accomplished by combining immunoelectrophoresis with autoradiography (Clausen and Munker, 1960). This combination of techniques allows the detection of specific proteins in minute amounts (Clausen, 1971).

VI. Plutonium and Proteins

Plutonium binding to proteins has been the object of much research in this country and England during the last few years. Knowledge of the chemical relationship between plutonium and proteins would facilitate better therapeutic and decorporation regimens after a plutonium exposure incident.

Much evidence has accumulated which indicates that transferrin is the principal plutonium carrying protein in serum (Stevens et al., 1965; Boocock and Popplewell, 1966; Turner and Taylor, 1968a; Bruenger et al., 1969). Stevens et al. (1968) also showed that some Pu can associate with albumin when incubated in vitro with canine blood. Chipperfield and Taylor (1968) showed that Pu is bound in bovine bone to sialo-proteins. Plutonium has been found bound to ferritin in the canine liver (Bruenger et al., 1969b); and in the rat liver (Boocock et al., 1970). Gomez (1973) presented some evidence that at least part of the plutonium in lymph is bound to transferrin.

The importance of bicarbonate ion in the binding of iron is well known (Aisen, 1967; Aisen, 1973) and is also implicated in the binding of Pu to transferrin (Turner and Taylor, 1968b).

The plutonium-transferrin complex is less stable than the iron-transferrin complex; Pu can be displaced by large excesses of iron (Turner and Taylor, 1968b). When compared to the plutonium-ferritin complex, the plutonium-transferrin complex is weaker (Bruenger, 1969).

Chemical form, particle size, route of exposure and individual biovariability have been previously enumerated as important factors to consider at the time of any plutonium exposure. They are also important in assessing the role of proteins in translocating plutonium (Bistline, 1973).

VII. Fission Track Analysis

Fission tracks result from molecular disturbances along the path of a heavy particle in a dielectric or insulating solid. The molecular disturbances are usually permanent and when a chemical is applied to the tracking material the disturbed molecular track is less resistant and will leave behind an etched track. The length and width of the track are characteristic of the particle under constant etching conditions. Thus, if the tracks left by a heavy particle are counted, they give an estimate of the size and quantity of the particular heavy particle incident upon the tracking medium.

The early work with fission track analysis was carried out by Price and Walker in the early sixties. They observed tracks in mica that were caused by fission fragments (Price and Walker, 1962). Further work showed that fission track method is a much more sensitive and rapid method for the detection of charged particles than such procedures as autoradiography. Price and Walker were later joined by R. L. Fleischer and have continued to be preeminent in the field (Piermattei, 1972).

Microanalysis of fission tracks has come into use recently. A dielectric is placed over the material being investigated and then the material is bombarded with neutrons. The resultant fission fragments are recorded by the dielectric. This allows the investigator to pinpoint the fragment location and activation analysis formulas were further developed to obtain concentration.

Using cellulose nitrate as the dielectric, Mory (1969) mapped the distribution of plutonium in fuel elements. Plastics have been used to map the distribution of Pu in biological specimens and show better resolution than photoemulsions (Becker and Johnson, 1970; Cole et al., 1970; Simons and Fitzgerald, 1970). Many new applications of this technique are being attempted in the field of biological research.

CHAPTER III

MATERIALS AND METHODS

I. Experimental Design

Two different chemical forms of plutonium were used in these experiments -- plutonium nitrate and plutonium oxide. Reactor-produced polydispersed plutonium oxide and plutonium nitrate were supplied by Rocky Flats Plant, Dow Chemical Company, Denver, Colorado. Monodispersed pure $^{239}\text{PuO}_2$ was supplied by the Inhalation Toxicology Research Institute, Albuquerque, New Mexico. Relevant characteristics of the plutonium compounds used are listed in Tables 2, 3, and 4.

Ten dogs were initially used to test canulation techniques, injection procedures and lymph collection time schedules. Then each chemical form and particle size was implanted in two different dogs; afferent lymph was collected from one dog and efferent lymph was collected from the other. Table 1 outlines the basic experimental design used in the studies. Due to cost factors, it was impossible to use more than one dog for each type of plutonium and lymph vessel.

II. Experimental Animals

A total of 20 adult dogs, 8 mongrels and 12 beagles, were obtained for use in this study. The dogs chosen weighed from 35-40

Table 1

Experimental Design

Dog No.	Chemical Form of Pu	Plutonium Characteristics	Lymph Vessel Collected
1	$\text{Pu}(\text{NO}_3)_4$	soluble	afferent
2	$\text{Pu}(\text{NO}_3)_4$	soluble	efferent
3	PuO_2	polydispersed	afferent
4	PuO_2	polydispersed	efferent
5	PuO_2	monodispersed .16 μ particles	afferent
6	PuO_2	monodispersed .16 μ particles	efferent
7	PuO_2	monodispersed .54 μ particles	afferent
8	PuO_2	monodispersed .54 μ particles	efferent
9	PuO_2	monodispersed 1.05 μ particles	afferent
10	PuO_2	monodispersed 1.05 μ particles	efferent

Table 2

Physical, Chemical and Isotopic Characteristics
of $\text{Pu}(\text{NO}_3)_4$ Supplied by Rocky Flats

$\text{Pu}(\text{NO}_3)_4$	Characteristics
Pu	= 269 g/ml
Total Alpha activity	= 20.2 MCi/ml
^{238}Pu	= 0.0098 wt %
^{239}Pu	= 93.810 wt %
^{240}Pu	= 5.796 wt %
^{241}Pu	= 0.309 wt % (9/73)
^{242}Pu	= 0.019 wt %
^{241}Pu	= 590 ppm based on Pu (9/73)

Table 3

Physical, Chemical and Isotopic Characteristics
of PuO₂ Supplied by Rocky Flats

PuO ₂	Characteristics
Pu	= 0.8826 g/g
Cation impurities	= 200 ppm (based on Pu)
An ²⁴¹	= 360 ppm based on Pu as of 10/1/70
An ²⁴¹ growth rate	= 15 ppm/month based on Pu
Pu ²³⁸	= 0.0105 wt %, based on Pu
Pu ²³⁹	= 93.898 wt %, based on Pu
Pu ²⁴⁰	= 5.695 wt %, based on Pu
Pu ²⁴¹	= 0.377 wt %, based on Pu as of 6/70
Pu ²⁴²	= 0.019 wt %, based on Pu
Atomic wt. of Pu	= 239.116 as of June, 1970
<u>Particle size (μ)</u>	
1 or less	68%
1 to 3	21%
3 to 5	4%
5 to 10	6%
10	1%

Mass Median Diameter Approximates 0.7

Table 4

PuO₂ Particle Sizes Supplied by Inhalation
Toxicology Research Institute

Segment [*] Number	Particle Size (μm)
2	1.05 ± 0.1 (SD)
7	0.54 ± 0.05
18	0.16 ± 0.02

* Segment number from Lovelace Aerosol Particle Separator System, Inhalation Toxicology Research Institute, Albuquerque, New Mexico.

pounds. The beagles came from the barrier-maintained colony of the Collaborative Radiological Health Laboratory. The mongrels were obtained as needed from the Colorado State University Experimental Animal Service and placed in quarantine for three weeks prior to inclusion in an experiment. The beagles were housed in the cage facilities of the Collaborative Radiological Health Laboratory. They were fed ad libitum* and watered with pans or an automatic watering system (licks) depending on the season. The dogs were observed daily and given regular veterinary care.

Six New Zealand white rabbits were used in the immunological studies. They were kept in temperature and air-controlled facilities at the Colorado State University Foothills Research Campus. They were fed ad libitum with rabbit pellets** supplemented by fresh greens. Daily observation and care was maintained during the course of the experiments.

All animals were maintained in accordance with provisions of the Laboratory Animal Welfare Act (1967).

III. Surgical and Implant Procedures

Each dog was given a preanesthetic injection of acepromazine, then anesthetized with an intravenous injection of pentobarbital

* Lab Dog Chow, Ralston Purina Co., St. Louis, Missouri.

** Ranchway Rabbit Pellets, Ranchway Feeds, Fort Collins, Colorado.

sodium. A continuous intravenous saline drip was started. The hair was clipped from the left hindlimb. (For efferent lymph collection the pelvic region was included.) A routine surgical scrub was performed and then the surgical site was cleaned with 70% ethyl alcohol and isolated with drapes.

For afferent lymph collection a 3 inch oblique skin incision was made parallel to the lateral saphenous vein as it crosses the tarsus. Fascia was bluntly dissected until the afferent lymphatics of the popliteal lymph node, running parallel to the lateral saphenous vein, were isolated. Usually two main lymph vessels were visible, one on each side of the lateral saphenous vein. Both vessels were ligated with 000 nylon. If any other lymph vessels were seen in the vicinity, they were also ligated. A pre-heparinized lymphangiography catheter^{*}, with a 25 gauge needle attached, was inserted in the larger lymphatic vessel and anchored with a 000 nylon ligature. The catheter was then connected to a test tube for lymph collection.

For efferent lymph collection a $3\frac{1}{2}$ inch incision was made parallel to the femoral artery as it transverses the femoral triangle. Fascia was dissected away and 2-4 efferent lymph vessels from the popliteal lymph node were seen running parallel to the femoral artery and vein. All lymph vessels were ligated with 000 nylon. The largest lymph vessel was found and a heparinized lymphangiography catheter,

^{*} Becton, Dickinson and Co., Rutherford, N. J.

with a 25 gauge needle attached, was inserted in the lymphatic and anchored with a 000 nylon ligature. The catheter was then connected to a test tube for lymph collection

Approximately a milliliter of lymph was collected before implant procedures were begun. Prior to injection the implant area was lined with plastic and absorbent paper as a precautionary measure. A continuous air sample was taken during implant procedures. Health physics personnel from Rocky Flats Plant of Dow Chemical Company provided monitoring services.

Plutonium nitrate was already in solution. Plutonium oxide, reactor-produced, was diluted with 2.0 ml of sterile saline. The monodispersed $^{239}\text{PuO}_2$ was diluted with a surfactant (Dowfax 2A1)* and placed in an ultrasonic waterbath for $2\frac{1}{2}$ hours to attain suspension. All plutonium solutions were shaken vigorously prior to implant.

A 22 gauge needle was positioned subcutaneously in the dorsal metatarsal area. It was checked for back flow of blood which would have indicated improper positioning of the needle. For the plutonium nitrate and reactor-produced plutonium oxide, 0.1 ml was withdrawn from the vial with 1.0 ml tuberculin syringe. The syringe was then attached to the pre-positioned needle and the plutonium injected. To obtain an easily detectable amount of plutonium, it was necessary to inject 1.0 ml of the suspended monodispersed $^{239}\text{PuO}_2$ particles.

* Dow Chemical Company, Midland, Mich.

The intervals for the collection of lymph following implant are listed in Table 5. After the four hour collection interval, the catheter was removed and the incision closed with 0 nylon. The animal was then euthanatized and disposed of by the Colorado State University radiation control office.

IV. Normal Lymph

Initially a 1.0 - 1.5 ml sample of lymph was collected from each dog to be used for control values.

Cell counts were obtained using the Unipet^{*} system and a Bright-line^{**} hemocytometer. An average of 3-4 counts were performed on each sample to obtain an accurate estimate for each dog. Smears were made from each sample, stained with modified Wrights-Giensa stain, and examined for morphology.

Protein determinations were made on each sample using the Lowry method (Lowry, 1951). Normal lymph samples were also run through each of the electrophoretic procedures (to be described in the section on plutonium-contaminated lymph) to give a basis for comparison of plutonium-induced changes.

^{*}Becton-Dickinson and Co., Rutherford, N. J.

^{**}AO Instrument Co., Buffalo, N. Y.

Table 5

Lymph Sample Collection Intervals

Fraction Number	Collection Interval
1	0-30 min
2	30-60 min
3	60-90 min
4	90-120 min
5	120-150 min
6	150-180 min
7	180-210 min
8	210-240 min

V. Plutonium Contaminated Lymph

A. Separation into Acellular and Cellular Fractions

Lymph samples from plutonium-exposed dogs were initially broken down into cellular and acellular fractions by centrifugation. Sample size was measured for later use in calculation of plutonium content.

Cellular fractions were counted for plutonium content using liquid scintillation procedures outlined in section V. F. Certain samples were designated for analysis using the fission track method described in Section VIII.

A 25 λ sample of acellular lymph was taken from each collected fraction for later analysis for plutonium by liquid scintillation techniques. The remaining acellular fraction was retained for biochemical analysis.

B. Discontinuous Gel Electrophoresis

The reagents to be used for discontinuous gel electrophoresis are listed in Table 6 along with reagent and buffer preparation. All reagents were mixed in advance and stored in the refrigerator at 4°C. Solution E was made fresh each day. Solution F was stored in an aluminum foil-covered container to prevent degradation due to exposure to light. Upper and lower buffers were used only once. The gel tubes were washed, soaked in a dichromate cleaning solution and dried before use.

Table 6

Discontinuous Gel Preparation

Resolving Gel			Stacking Gel		
Solution A	1 NHCL	24.0 ml	Solution B	1 H ₃ PO ₄	12.80 ml
	Tris	18.15 g		Tris	2.85 g
	Temed	0.4 ml		Temed	0.10 ml
	H ₂ O to	100 ml		H ₂ O to	100 ml
Solution C	Acrylamide	30.0 g	Solution C	Acrylamide	5.0 g
	Bis	0.8 g		Bis	1.25 g
	H ₂ O to	100 ml		H ₂ O to	100 ml
Solution E	Ammonium Persulfate	0.03 g	Solution F	Riboflavin	0.002 g
	H ₂ O to	100 ml		H ₂ O to	100 ml

Resolving Gel Mixing Ratio

A	C	E
1	1	2

Stacking Gel Mixing Ratio

B	D	F
1	2	1

Upper Buffer

Tris	6.42 g
Glycine	3.98 g
H ₂ O to	1 liter

Lower Buffer

Tris	24.20 g
Made up to	2 liters
with 0.05 N HCL	

The resolving gels were made 6.8 cm long. Gel tubes were covered by two layers of Parafilm^{*} and placed in a level polymerization rack. The gel mixture was prepared and placed in the gel tubes. They were overlaid with 0.5 cm of water to prevent meniscus formation. The gels were allowed to chemically polymerize for at least one hour.

The water overlayer was shaken from all tubes and dried further with cotton swabs. The stacking gel mixture was prepared as outlined in Table 6 and a 3 cm layer was added to the gel tubes with a syringe. Again the gel tubes were overlaid with 0.5 ml of water. The tubes were placed between two fluorescent polymerization lamps for at least one hour to allow photopolymerization to take place.

The gels were placed in a Hoeffer^{**} gel electrophoresis apparatus connected to an Isco^{***} power supply. The lower buffer was placed in the electrophoresis tank and the tank was leveled. Fifty λ of 50% sucrose was placed over each gel. A 25 λ sample of lymph was placed on top of the sucrose. Bromophenol blue was used as the marker dye in one control gel.

The gels were run at 0.5 ma per tube for 0.5 hour, 1.0 ma per tube for 1.0 hour, and 2.0 ma per tube until the marker dye started coming off the bottom of the tube.

^{*}American Can Company, Neenah, Wisconsin.

^{**}Hoeffer Scientific Instruments, San Francisco, Calif.

^{***}Instrumentation Specialties Co., Lincoln, Nebr.

Gels were stained, frozen or sectioned immediately for liquid scintillation counting. The gels to be stained were placed in either 0.05% coomassie blue in 20% trichloroacetic acid (TCA) or a 10% acid fast green solution in 10% acetic acid. Coomassie blue stained gels were destained in 20% TCA, while gels stained with acid fast green were destained in 10% acetic acid. Gels to be frozen were placed in a acetone-dry ice bath and then stored in a freezer. The remaining gels were sectioned immediately with the gel cutting apparatus shown in Figure 1. Sections of approximately 2.0 mm were cut. Each section was placed in a glass liquid scintillation vial and 1.5 ml of 30% hydrogen peroxide was added to digest the gel for later counting.

C. Isoelectric Focusing Gel Electrophoresis

The details of reagent and buffer preparation used for isoelectric focusing gel electrophoresis are shown in Table 7. All reagents were prepared in advance and stored in the refrigerator at 4°C except ampholines* which were kept frozen. Solution C was stored in an aluminum foil-covered container to prevent degradation due to exposure to light. Upper and lower buffers were used only once. The gel tubes were washed, soaked in a dichromate cleaning solution and dried before use.

The bottom of each gel tube was covered with Parafilm and placed in a polymerization rack. The gel mixture was added to a

* LKB Products, Stockholm, Sweden.

Figure 1. Gel sectioning instrument with probe for removal of gel sections.

50% COTTON
ENGLISH BOND
by
FOX RIVER

50% COTTON

Table 7

Isoelectric Focusing Gel Preparation

Solution A	Temed	0.8 ml
	H ₂ O to	100 ml
Solution B	Acrylamide	30.0 g
	Bis	0.8 g
	H ₂ O to	100 ml
Solution C	Riboflavin	6 mg
	H ₂ O to	100 ml
Solution D	40% Ampholine (pH 3-10)	2.5 ml
	H ₂ O to	100 ml

Gel Mixing Ratio

A	B	C	D
1	2	1	4

Upper Bufferconcentrated H₂SO₄ 2 mlH₂O to 1 literLower Buffer

Monoethanolamine 16 ml

H₂O to 1 liter

height of 7.0 cm with a syringe and over-layered with 0.5 cm of water. Photopolymerization was allowed to take place between two fluorescent polymerization lamps for at least one hour.

The water was removed and the gels put into a Hoeffer gel electrophoresis apparatus. The lower buffer was placed in the electrophoresis tank. Fifty λ of 50% sucrose was added to each gel. Twenty-five λ of the lymph sample was carefully placed on top of the sucrose followed by a small layer of 2% ampholines to prevent mixing while adding the upper buffer. The gels were electrophoresed at a constant 100 V for one hour and then 200 V for 4.5 hours.

Gels were stained, frozen, or sectioned immediately for liquid scintillation counting and pH determination. The gels to be stained were first put in 20% TCA to remove ampholines. The TCA was changed several times over a two day period. Gels to be stained were placed in a 0.05% Coomassie blue stain for 24 hours and then destained in 20% TCA. Gels to be frozen were placed in an acetone-dry ice bath and then stored in a freezer. Gels were sectioned with the apparatus shown in Figure 1. Those selected for liquid scintillation counting were placed in glass liquid scintillation vials with 1.5 ml of 30% hydrogen peroxide to digest the gels. Gels sectioned for pH determinations were placed in 3.0 ml test tubes and 1.0 ml of

double-distilled, deionized water was added. They were allowed to set over night and then were read with a pH meter.*

D. Cellulose Acetate

Lymph samples were electrophoresed using cellulose acetate as the support medium. The procedure used was that of Gelman (1970). Twelve λ of lymph was added to each cellulose acetate strip. The strips were then placed in a Gelman** electrophoresis chamber at 300 V for one hour and fifteen minutes. Duplicate samples were run, one for staining with Ponceau Red stain for protein location and identification, the other for sectioning into 0.5 cm pieces. The sections were then deposited in separate liquid scintillation vials and 5.0 ml of concentrated nitric acid was added to dissolve the cellulose acetate. The liquid scintillation procedure, as described in Section V. F., was then used.

Stained cellulose acetate strips were scanned with a Gelman** Densitometer and percentages of particular protein groups determined, e.g., albumin, α -globulin, β -globulins, and γ -globulins.

* Fischer Scientific Co., St. Louis, Mo.

** Gelman Instrument Co., Ann Arbor, Mich.

E. Immunoelectrophoresis

Antibodies against lymph proteins were produced in rabbits using the immunization procedures outlined in Table 8 and developed by Bier (1972). Blood obtained from the immunized rabbits was allowed to clot, centrifuged and serum collected. Serum titers were determined against lymph proteins following each bleeding. Serum samples were then divided into 1.0 ml volumes and frozen at -20°C until use.

The immunoelectrophoresis procedure used was that of Scheidegger (1955) except for a change in the buffer. The buffer used instead contained 20.6 g of sodium barbital in 1.0 liter of double-distilled water with the pH adjusted to 8.2 with concentrated hydrochloric acid (Williams and Chase, 1971). Duplicate samples were run on all lymph. One sample was stained with Thiazine Red dye. The other was dried for autoradiography. Autoradiography was performed using NTB2^{*} emulsion.

F. Liquid Scintillation Counting

Disc gel, isoelectric focusing gel, and cellulose acetate sectioned samples were placed on hot plates at 180°C until all hydrogen peroxide and nitric acid were evaporated. These samples were then ready for addition of the cocktail and liquid scintillation

^{*}Eastman Kodak Co., Rochester, N. Y.

Table 8

Immunization Procedure

Day 1	15.0 mg of antigen per 1.0 ml saline. 0.5 ml antigen solution injected intravenously 0.5 ml antigen solution + 0.5 ml saline and 1.0 ml complete Freund's Adjuvant* mixed. Then 0.3 ml injected subcutaneously in 5 areas of the back and in each front foot pad.
Day 8	15.0 mg of antigen per 1.0 ml saline. 1.0 ml antigen solution + 1.0 ml Complete Freund's Adjuvant* injected subcutaneously in 5 areas of the back.
Day 15	15.0 mg of antigen per 1.0 ml saline. 1.0 ml antigen solution + 1.0 ml Incomplete Freund's Adjuvant* injected subcutaneously in 5 areas of the back.
Day 30	15.0 mg of antigen per 1.0 ml saline. 1.0 ml antigen solution + 1.0 ml Incomplete Freund's Adjuvant* injected subcutaneously in 5 areas of the back.
Day 42	Bleed ear vein 10.0 cc
Day 56	Bleed ear vein 10.0 cc

* Difco Laboratories, Detroit, Mich.

α -counting using a Nuclear Chicago Mark II^{*} liquid scintillation counter.

Plutonium contaminated cell and protein samples were processed by the Keough and Powers (1970) procedure for liquid scintillation α -counting.

G. Gel Scanning Procedures

Gels were scanned using a Vicont^{**} linear gel scanner. Quartz cuvettes were used to scan gels at 280 nm to locate protein peaks prior to sectioning, in order to be able to correlate sections with stained gels and protein recovery. Coomassie stained gels were scanned at 530 nm and acid fast green stained gels were scanned at 640 nm. All scans were recorded with a Sargent Welch Model SRLT^{***} recorder for later use.

VI. Control Protein Studies

A. Time Studies

Time studies, using lymph protein contaminated with $\text{Pu}(\text{NO}_3)_4$, were conducted to determine changes during electrophoresis in plutonium-protein relationships.

* Nuclear Chicago, Des Plaines, Ill.

** Brinkmann Instruments, Westburg, N. Y.

*** Sargent Welch Co., Denver, Colo.

The discontinuous system used was the same as that described in Table 6. Twenty-five λ samples of plutonium-contaminated lymph were taken from a single tube so that a constant amount of plutonium and protein would be added to each gel. Once the marker dye reached the resolving gel, duplicate gel samples were removed from electrophoresis every 15 minutes for 1.5 hours. One sample was stained with Coomassie blue dye and the other was sectioned for liquid scintillation counting. The duplicate samples were handled in this manner for comparison of protein location. Each section was placed in a glass liquid scintillation vial and 1.5 ml of 30% hydrogen peroxide added. Liquid scintillation was carried out as stated in Section V. F.

The isoelectric focusing system used was the same as that in Table 7. The isoelectric focusing gel time study was carried out in the same manner as for the discontinuous system except that duplicate samples were taken every hour for six hours after electrophoresis began.

B. Standard Proteins

Several known protein samples were compared with lymph proteins. The proteins used for comparison include canine albumin^{*},

^{*}Sigma Chemical Co., St. Louis, Mo.

canine α , β , and γ -globulin^{*}, canine transferrin^{**}, equine transferrin^{***}, and ovatransferrin^{****}. They were run through both disc and isoelectric focusing gel electrophoresis. The information was used for comparison as to location and identification of lymph protein groups which were also run through the disc and isoelectric focusing gel systems.

VII. Preparative Gel Column

A preparative polyacrylamide gel column^{*****} was used to separate 40 mg of pooled lymph proteins (Figure 2). The polyacrylamide preparation used was the same one as outlined in Table 6. Fifteen ml of stacking gel and 40 ml of resolving gel were used in the column. One ml of 50% sucrose was initially added to the column, followed by one ml of bromophenol blue marker dye, and the 40 mg of pooled lymph proteins.

The column was run at a constant 10.0 ma until the marker dye was through the stacking gel and then 30 ma until the last protein peak came off the column.

^{*} Mann Research Labs., Orangeburg, N. Y.

^{**} Miles Laboratories, Kankakee, Wisc.

^{***} National Biochemical Corp., Cleveland, Ohio.

^{****} Dr. P. Azari, Colorado State University, Fort Collins, Colo.

^{*****} Shandon Scientific Company Limited, London, England.

The flow rate through the collecting chamber was 12 ml per hours. Upper buffer was used to elute the proteins off the column. The eluent off the column was checked with a Uvicord* ultraviolet monitoring system set at 280 nm. Samples were collected in one ml fractions and all tubes were read at 280 nm to get the final elution profile.

Peak samples, as seen in Figure 11, were pooled and dialyzed, using dialysis membranes with a molecular weight cutoff of 12,000, against distilled water. The peak samples were then freeze-dried and analyzed with discontinuous and isoelectric focusing gel systems previously described.

VIII. Fission Track Analysis

Lymph cells selected for analysis by the fission track method were collected by passage through a Millipore filter** with a pore size of 0.45 μ . The filters were dried and taken to the Rocky Flats Plant of Dow Chemical Company, Denver, Colorado for analysis.

The sample surface was placed in intimate contact with polycarbonate film. Sample and film were sandwiched between two sheets of polyethylene. The sandwiches were then rolled into a cylinder (tight enough to maintain contact between the sample and the

* LKB Products, Stockholm, Sweden.

** Millipore Corporation, Bedford, Mass.

polycarbonate detector film). Tape was used to hold the materials in the cylinder shape.

The sample was then inserted into a four dram polyvial, which was then heat sealed. The sealed vial was placed inside a standard polyethylene TRIGA* tube for irradiation. The irradiation was carried out in the lazy susan section of the TRIGA* reactor.

Samples containing polydispersed PuO_2 particles were irradiated for 100 minutes at 100 kilowatts (KW) power. These conditions yielded an integrated thermal neutron dose of 1.45×10^{15} neutrons per cm^2 . Under these conditions, particles 0.16μ in diameter yield about 50 tracks per alpha star. Monodispersed particles of known size were irradiated under different conditions. For example, 1μ size particles were irradiated for three minutes at 10 KW. The reactor parameters were selected to optimize the number of tracks per particle, 5 to 50 tracks being ideal for counting purposes.

After irradiation, time was allowed for the short-lived nuclides to decay out and then the film was removed. The film was etched in 6 N NaOH heated to 60°C for 15 minutes, then washed in dilute acid (HCL) to stop the etching and finally washed with distilled water, dried and mounted on a microscope slide for track counting. Optical magnification used was 125X.

* United States Geological Survey, Denver, Colo.

The Murri equation was used to calculate particle size (Hayden et al., 1970).

$$d = \frac{6MR_+}{\phi\sigma N_o\pi\rho ft}^{1/3}$$

where:

d = particle size diameter (cm)

M = molecular weight

R_+ = track count divided by detector efficiency, geometry
and the number of particles per event.

ϕ = neutron flux

σ = fission cross section

N_o = Avagodroes number

ρ = density

f = number of fissile atoms in the compound

t = time

CHAPTER IV

RESULTS AND DISCUSSION

I. Lymph Constituents

A. Lymph Flow

The volume of lymph collected following plutonium implants is shown in Table 9. For afferent lymph the total volume ranged from 4.5 ml to 7.5 ml, while the total volume for efferent lymph ranged from 4.5 ml to 8.5 ml for the 4.0 hour collection period. These values are similar to those found by Smith et al. (1970) in sheep. Lewis and Winsey (1970) observed a flow rate of 1.4 ml per hour in a 2.5 kg cat. When weight is taken into account, 2.5 kg for the cat versus an average of 17 kg for the dogs, the values obtained are reasonable.

No large differences are seen between afferent and efferent lymph flow. The lymph flow in each dog was consistent during each 0.5 hour collection interval, although there was variability from dog to dog. Thus implantation of plutonium did not increase lymph flow over the 4.0 hour collection period.

B. Lymph Cells

The lymph cell counts for the different dogs used in the plutonium implant experiments are shown in Table 10. All cell counts

Table 9

Total Lymph Volume (ml) Collected in 0.5 Hour
Intervals Following Plutonium Implants

Dog No.	Elapsed Hours After Implant								Total for 4.0 Hours
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	
<u>Afferent</u>									
1	1.2	1.1	0.9	0.6	0.4	1.0	1.0	1.0	7.2
3	0.8	0.8	0.6	1.0	0.9	0.9	0.6	0.5	5.9
5	0.7	0.6	0.8	0.4	0.5	0.9	0.7	1.3	5.9
7	0.7	0.5	0.5	0.4	0.5	0.5	0.6	0.6	4.5
9	0.7	0.6	0.6	0.6	0.5	0.7	0.4	0.8	4.9
<u>Efferent</u>									
2	1.1	0.9	1.0	1.0	0.8	1.2	1.4	1.1	8.5
4	0.7	0.6	0.8	0.5	0.6	0.6	0.8	0.8	5.4
6	0.7	1.3	1.0	1.2	0.5	1.1	0.8	0.7	7.3
8	0.5	0.6	0.6	0.6	0.7	0.7	0.6	0.9	4.5
10	0.6	0.6	0.4	0.7	0.6	0.7	0.5	0.8	4.9

Table 10

Lymph Cell Counts

Dog No.	Plutonium implanted	Cells per mm ³
<u>Afferent pre-implant</u>		
1	$\text{Pu}(\text{NO}_3)_4$	110
3	polydispersed PuO_2	220
5	0.16 μ particles PuO_2	220
7	0.54 μ particles PuO_2	110
9	1.05 μ particles PuO_2	440
	Average	220
<u>Efferent pre-implant</u>		
2	$\text{Pu}(\text{NO}_3)_4$	525
4	polydispersed PuO_2	23,595
6	0.16 μ particles PuO_2	2,640
8	0.54 μ particles PuO_2	1,320
10	1.05 μ particles PuO_2	8,910
	Average	7,398

were made before implantation. The average cell count for afferent lymph was 220 cells per mm^3 , while the average cell count for efferent lymph was 7398 cells per mm^3 . Yoeffy and Drinker (1939) found 0 to 800 cells per mm^3 in afferent peripheral lymph in sixteen dogs. In 1970 Smith et al. counted 200-700 cells per mm^3 in afferent lymph of sheep and 300-1000 cells per mm^3 in efferent lymph of the popliteal lymph node. These results indicate that the lymph cell counts obtained from the dogs in these experiments were within the normal range.

The different cell types seen in efferent lymph are pictured in Figures 3A, 3B, and 3C. Spindle-shaped lymphocytes, as shown in Figure 3A, were commonly seen in efferent lymph but not in afferent lymph. Occasionally dividing lymphocytes, Figure 3B, were seen in efferent lymph. A few phagocytic cells were present, but 99% of the cells were either medium-sized or large lymphocytes.

Typical cells seen in afferent lymph are shown in Figures 3D, 3E, and 3F. Phagocytosis of PuO_2 particles is a common phenomenon seen with phagocytic cells (Sanders, 1970; Dagle, 1973). The microphage in Figure 3E, with the phagocytosed particles, was from a dog injected with PuO_2 . It is conceivable that the particles within the cell were PuO_2 particles. Active phagocytic cells comprised 2-5% of the cell population of afferent lymph, while small lymphocytes made up

Figure 3. Lymph cells. X1200

- A. Spindle-shaped lymphocyte found in efferent lymph.
- B. Lymphocyte dividing in efferent lymph.
- C. Lymphocyte seen in efferent lymph.
- D. Active macrophage seen in afferent lymph.
- E. Microphage with phagocytosed particles (arrow) seen in plutonium contaminated afferent lymph.
- F. Lymphocyte seen in afferent lymph.

the rest. The presence of phagocytic cells further emphasizes their availability for the movement of plutonium in lymph.

The various sizes of lymphocytes seen in afferent and efferent lymph are illustrated in Figure 4. Efferent lymphocytes were usually larger than afferent lymphocytes, indicating that the efferent lymphocytes were younger (Schalm, 1971).

C. Lymph Protein Concentration

The total lymph protein concentration (mg/ml) is given in Table 11. Lymph protein concentrations prior to plutonium implant, 1.0 hour post-implant and 4.0 hours post-implant are listed. No significant difference was seen between the protein concentration means before implantation, 1.0 hour post-implant, and 4.0 hours post-implant in both afferent and efferent lymph. Absence of a significant change indicates either that the plutonium insult was not injurious enough to cause a change in lymph proteins or that a delayed reaction occurred which was not detectable in the short 4.0 hour collection period. Lewis (1967) reported similar results in the lymph protein pattern following low level heat injury.

The pre-implant protein concentration for afferent lymph was 13.81 ± 4.02 mg/ml, while the pre-implant protein concentration for efferent lymph was 21.43 ± 2.80 mg/ml. Using a student's t-test (Snedecor and Cochran, 1967) the means were found to be significantly different at the 0.05 level. Yoeffey and Courtice, in 1970, reported

Figure 4. Lymph cells. X1200

A, B, C. Various sizes of lymphocytes seen in efferent lymph.
D, E, F. Various sizes of lymphocytes seen in afferent lymph.

Table 11

Total Lymph Protein Concentration (mg/ml)

Dog No.	Plutonium implanted	control pre-implant	1.0 hour post-implant	4.0 hours post-implant
<u>Afferent</u>				
1	$\text{Pu}(\text{NO}_3)_4$	15.88	13.32	14.87
3	polydispersed PuO_2	10.00	10.85	9.95
5	0.16 μ particles PuO_2	19.85	19.61	15.68
7	0.54 μ particles PuO_2	11.31	12.67	13.18
9	1.05 μ particles PuO_2	12.03	11.69	14.85
		<u>13.81$\pm$4.02</u>	<u>13.63$\pm$3.47</u>	<u>13.71$\pm$2.29</u>
<u>Efferent</u>				
2	$\text{Pu}(\text{NO}_3)_4$	17.60	21.80	18.90
4	polydispersed PuO_2	24.05	19.95	18.65

Table 11 (continued)

Dog No.	Plutonium implanted	control pre-implant	1.0 hour post-implant	4.0 hours post-implant
6	0.16 μ particles PuO ₂	20.50	23.62	17.82
8	0.54 μ particles PuO ₂	20.66	18.53	19.87
10	1.05 μ particles PuO ₂	24.34	23.86	21.21
		<u>21.43$\pm$2.80</u>	<u>21.55$\pm$2.31</u>	<u>19.29$\pm$1.30</u>

that protein concentration in lymph varies with the location of the lymph vessel. They found that protein concentration was usually slightly higher on the efferent side of a lymph node. The data obtained in this experiment for the afferent and efferent lymph vessels of the popliteal lymph node followed this trend.

Protein concentration is an important factor to consider when comparing plutonium binding in serum and lymph. In the lymph there is a reduced number of binding sites due to a lower concentration of proteins. This will be discussed further in the section on plutonium-contaminated lymph.

II. Biochemical Studies of Normal Lymph

A. Standard Proteins

To identify the proteins to which plutonium is bound, it is necessary to know the location of specific proteins following electrophoretic separation. Commercially available standard proteins were compared, by discontinuous gel electrophoresis, with normal lymph proteins to determine the location of specific protein groups in lymph.

The location of transferrins from different species, along with canine afferent lymph, are illustrated in Figure 5. This comparison allows the transferrin bands in canine afferent lymph to be identified after being electrophoresed on discontinuous gels. The location of transferrin, identified in the discontinuous gel system, differs considerably from the position indicated by Gomez (1973). Instead of

Figure 5. Discontinuous gels

- A. Normal canine afferent lymph with arrow indicating transferrin.
- B. Purified canine transferrin (arrow).
- C. Purified equine transferrin (arrow).
- D. Purified ovatransferrin (arrow).

Figure 6. Discontinuous gels

- A. Canine albumin (bottom arrow) and α -globulins (upper two arrows).
- B. Canine β -globulins (between arrows).
- C. Canine γ -globulins (between arrows).
- D. Normal canine afferent lymph (top arrow indicating γ -globulins, middle arrow indicating canine transferrin, and bottom arrow indicating canine albumin).

A

B

C

D

A

B

C

D

conducting control protein studies, he used published electrophoretic patterns of serum proteins for comparison with lymph proteins to identify the transferrin band. This can lead to discrepancies because of differences in discontinuous gel systems.

Further comparative protein studies are pictured in Figure 6. Four known canine serum protein groups (albumin, α -globulin, β -globulin, and γ -globulin) are matched against canine afferent lymph. Excess afferent lymph protein was electrophoresed on gel D to allow a visualization of even the weakest band. Twenty-one different protein bands were seen on this gel. Winsey (1972), using starch gel electrophoresis, showed that in afferent lymph 19 separate bands of protein could be demonstrated; thus the resolution achieved with the discontinuous gel system enabled the detection of two additional protein bands.

B. Afferent versus Efferent Lymph

Afferent lymph differs from efferent lymph quantitatively in that it has a lower protein concentration (as demonstrated in section I. C. of this chapter). To investigate whether a qualitative difference occurs, afferent and efferent lymph samples were electrophoresed on both discontinuous and isoelectric focusing gel systems. The resultant gels, along with the pH gradient for the isoelectric focusing gels, are pictured in Figures 7 and 8. Little difference was seen in the afferent and efferent lymph protein bands using these two systems.

D

Figure 7. Discontinuous gels

A. Normal canine afferent lymph (upper arrow indicating transferrin and lower arrow indicating albumin).

B. Normal canine efferent lymph (upper arrow indicating transferrin and lower arrow indicating albumin).

Figure 8. Isoelectric focusing gels with pH gradient shown

A. Normal canine afferent lymph (upper arrow indicating albumin and lower arrow indicating transferrin).

B. Normal canine efferent lymph (upper arrow indicating albumin and lower arrow indicating transferrin).

The lymph of most dogs followed this pattern; however, two dogs had aberrant efferent lymph protein patterns. Their electrophoretic patterns are compared with that of a normal dog in Figures 9 and 10. The differences are greatest in the β and γ -globulins where the intensity of the bands for these two protein groups is at odds with the normal electrophoretic intensity pattern. Genetic differences, as well as immunologic competence, could result in the changes observed in the electrophoretic patterns of these two dogs (Feeney and Allison, 1969).

C. Preparative Gel Column

To compare the electrophoretic migration patterns of specific lymph proteins on both discontinuous and isoelectric focusing gel systems, it was necessary to initially isolate proteins into groups. This was done using a preparative gel column.

Optical density readings (280 m μ) were made on fractions collected from the preparative gel column to determine protein content. The results are plotted in Figure 11 and peaks were indicated. The peaks contained different lymph protein groups that could be used in the comparison of the gel systems. Fractions making up each peak were pooled and electrophoresed on both discontinuous and isoelectric focusing gel systems. Photographs of the disc gels are shown in Figures 12 and 13. Peak 12 is missing because the bands were too faint to be recorded. The peak number and the pH

Figure 9. Discontinuous Gels. Upper arrow indicates transferrin and the lower arrow indicates albumin in each of the gels.

- A. Aberrant canine efferent lymph.
- B. Aberrant canine efferent lymph.
- C. Normal canine efferent lymph.

Figure 10. Isoelectric focusing gels. Upper arrow indicates transferrin and the lower arrow indicates albumin in each of the gels.

- A. Aberrant canine efferent lymph.
- B. Aberrant canine efferent lymph.
- C. Normal canine efferent lymph.

A

B

C

A

B

C

1111

Figure 11. Optical density readings (280 m μ) on fractions collected from the preparative gel column. Peaks are numbered 1 through 12.

Figure 12. Discontinuous gels. Peaks 1 through 6 collected from the preparative gel column. Arrows indicate the most intense protein band from each peak electrophoresed on each gel. The arrow in gel 1 indicates albumin.

Figure 13. Discontinuous gels. Peaks 7 through 11 collected from the preparative gel column. Arrows indicate the most intense protein band from each peak electrophoresed on each gel. The arrow in gel 10 indicates transferrin. Gel 12 is normal canine afferent lymph.

1

2

3

4

5

6

7

8

9

10

11

12

range observed in the isoelectric focusing gels are shown in Table 12. Based on the migration patterns of known standard proteins and isoelectric points it was concluded that peak 1 contained albumin and peak 10 transferrin. Both gel systems produced the same results for the identification of the specific proteins in these two peaks; therefore, it is reasonable to use the two gel systems for comparison of specific lymph proteins.

D. Time Studies

The plutonium-protein bond has been described by Taylor (1973) as very weak. Bruenger et al. (1969) showed that much of the serum protein bound plutonium dissociated during electrophoresis and was spread over the length of the gel. Only a small plutonium peak was associated with the transferrin bands. However, Bruenger used an in vitro system to obtain serum protein bound plutonium and sectioned gels into only 16 parts. Gómez (1973), electrophoresing plutonium contaminated lymph proteins, sectioned gels into only three parts and looked at only major protein groups (γ -globulins, β -globulins, α -globulins and albumin). No accounting of dissociated plutonium was made. Neither researcher investigated what happened to the peaks during actual electrophoresis. To evaluate the meaning of plutonium peaks found on both discontinuous and isoelectric focusing gels, an experiment, using paired samples, was performed with each gel system.

Table 12

pH Range of Protein Peaks from the
Preparative Gel Column

Peak No.	pH Range
1	4.5 - 4.9
2	4.0 - 4.2
3	4.1 - 4.4
4	4.3 - 4.7
5	4.1 - 4.6
6	4.4 - 4.8
7	5.0 - 5.3
8	5.2 - 5.7
9	5.6 - 5.8
10	5.6 - 5.9
11	6.5 - 6.9
12	6.8 - 7.3

1. Discontinuous gel electrophoresis

The stained discontinuous gels that resulted from the time study of plutonium contaminated lymph proteins are shown in Figure 14. Their sectioned counter parts, containing a fraction of plutonium in each section, are plotted in Figure 16. Two major Pu peaks were seen during the protein separation in the resolving gel. Both peaks spread over more sections and diminish in height with time. This is especially true of the first peak. At 90 minutes peak 1 spreads from section 8 to section 12, while peak 2 spreads from section 29 to section 36.

Previous studies with standard proteins indicated that peak 1 corresponded with the location of transferrin and peak 2 with albumin.

According to Young and Perkins (1968) transferrin will bind molecules of iron and can exist in three biochemical states; two, one or no molecules of iron bound to a transferrin molecule. Since the binding of iron to transferrin causes a conformational change in the transferrin molecule, each transferrin state may electrophorese differently, resulting in the spread of transferrin molecules on a gel (Luk, 1971; Aasa, 1972). This phenomenon accounts for the spreading of the plutonium peak associated with transferrin that was observed in this experiment. Diminution of the plutonium peak is the result of a stripping effect whereby plutonium molecules are removed from transferrin by the electrical field that exists during electrophoresis.

Figure 14. Discontinuous gels. Time study, with time = 0 being when the plutonium contaminated proteins entered the resolving gel.

Figure 15. Isoelectric focusing gels. Time study, with time = 0 being the initiation of electrophoresis of the plutonium contaminated proteins.

15 min.

30 min.

45 min.

60 min.

75 min.

90 min.

1.0 hr.

2.0 hr.

3.0 hr.

4.0 hr.

5.0 hr.

6.0 hr.

Figure 16. Fraction of Pu detected in sections of discontinuous gels at specific times after the proteins entered the resolving gel.

Section No.

Section No.

Also the slow movement of transferrin molecules in the gel may allow for more competitive binding of other ions to Pu, causing the plutonium-transferrin bond to break. This would cause an even further reduction in the height of the plutonium peak.

Albumin makes up a large percentage of the total lymph proteins (60-70%); thus, the mass of albumin alone caused spreading of albumin molecules over several sections. Part of the ion front in the gel overlaps albumin and may contribute some plutonium molecules to the albumin peak. Plutonium associated with the ion front was reported by Bruenger et al. (1969). The decrease in the Pu peak associated with the albumin bands is undoubtedly due to a stripping effect such as was observed with transferrin molecules. However, because albumin moves much faster on the gel the stripping effect is minimized.

This time study indicated that plutonium peaks, associated with specific proteins, can be determined but that accurate estimates of the percentage of Pu binding to a particular protein would be difficult due to the stripping effect and to the competitive binding of other ions to plutonium occurring at the pH of this gel system.

2. Isoelectric focusing gel electrophoresis

The stained isoelectric focusing gels that resulted from the time study of plutonium contaminated lymph proteins are shown in Figure 15. Their sectioned counterparts, containing a fraction of Pu in each section, are plotted in Figure 17. Three major

Figure 17. Fraction of Pu detected in sections of isoelectric focusing gels at specific times after electrophoresis initiated.

Pu peaks are consistently seen as the proteins are separated in the resolving gel. Again the peaks spread and diminish in height with time, especially the first two peaks. At 6.0 hours peak 1 spreads from section 1 to 4, peak 2 spreads from section 8 to 11, and peak 3 spreads from section 17 to 25.

Peak 1 did not correspond to the location of any protein and was found to be a plutonium precipitate that occurred when the plutonium contaminated lymph was applied to the gels. Previous studies with standard proteins indicated that peak 1 corresponded with the location of albumin and peak 2 with transferrin.

Each of the transferrin states has a different isoelectric point and causes a spreading effect on the gels since they contain a pH gradient. The height of the Pu peak associated with transferrin decreases slightly with time but the stripping effect is not as severe as with the disc gels.

The mass of the albumin fraction again causes a spreading effect. The plutonium peak associated with albumin is severely reduced in height with time. The albumin quickly reaches its isoelectric point in the gels and remains stationary. This causes a much greater stripping effect than was seen with the disc gels.

The isoelectric focusing gel time study indicates that the same problems exist as with the disc electrophoresis system. Identification of specific proteins involved in plutonium binding can be achieved but

the problems outlined preclude an accurate estimate of the percentage of plutonium binding to an individual protein by the methods used in this study.

III. Plutonium Contaminated Lymph

A. Acellular versus Cellular Lymph Fractions

Two processes are involved in the transport of plutonium in lymph -- the cellular process which involves the phagocytosis of plutonium particles and the acellular process which binds plutonium to proteins or other biological macromolecules. Investigation of the amounts of plutonium moving in these two processes is important in devising therapeutic regimens for plutonium contaminated wound incidents.

Using polydispersed PuO_2 , Gomez (1973) showed that 65% of the plutonium moved in the cellular fraction. However, the ratio of acellular to cellular plutonium may change with time as dissolution of Pu particles occurs. Gomez did not report the time post-implant of his estimate.

1. Soluble versus insoluble plutonium

Soluble and insoluble forms of plutonium are initially handled very differently by the body when they gain entry through contaminated wounds (Bistline, 1973; Johnson, 1969). To investigate the differences, a soluble form of plutonium ($\text{Pu}(\text{NO}_3)_3$) and an insoluble form (PuO_2) were chosen. The values for the fraction of initial

implant dose of plutonium found in lymph for 0.5 hour collection intervals are given in Table 13 for each dog and type of plutonium. No attempt was made to fit data to regression curves because only one dog was used for each set of conditions.

The acellular and cellular fractions of afferent lymph were plotted (Figure 18) from a dog implanted with $\text{Pu}(\text{NO}_3)_4$. It was found that the fraction of implant dose was a factor of a 1000 greater in the acellular fraction. This is consistent with what is expected with ionic forms of plutonium (Turner and Taylor, 1968a). No significant change in the acellular or cellular fraction of plutonium was seen with time.

The data for efferent lymph, from a dog implanted with $\text{Pu}(\text{NO}_3)_4$, is presented in Figure 19. It showed that the acellular fraction contained 50 times as much plutonium as the cellular fraction as judged from the fraction of implant dose parameter. This is again consistent with what is expected with ionic forms of plutonium (Turner and Taylor, 1968a). As with afferent lymph, significant changes in cellular and acellular plutonium concentration were not seen with time.

As plutonium is translocated in lymph it eventually reaches a lymph node, where it can be phagocytosed by macrophages, pass into the vascular system via lymph-venous communications, or leave the node through efferent lymph vessels. Comparison of acellular and cellular lymph fractions is important in determining Pu movement in afferent and efferent lymph. Contrasting plutonium transport in

Table 13

Fraction of Initial Implant Dose of Plutonium

Dog No.	Plutonium Characteristics	Lymph Characteristics	Elapsed Hours After Implant							
			0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
1	$\text{Pu}(\text{NO}_3)_4$	afferent acellular	1.1935×10^{-3}	1.9159×10^{-3}	2.0834×10^{-3}	2.6108×10^{-3}	1.9004×10^{-3}	3.5432×10^{-3}	3.8962×10^{-3}	4.2681×10^{-3}
1	$\text{Pu}(\text{NO}_3)_4$	afferent cellular	1.0201×10^{-6}	1.6036×10^{-6}	3.3851×10^{-6}	2.7895×10^{-6}	2.6831×10^{-6}	1.4641×10^{-6}	3.9533×10^{-6}	2.8324×10^{-6}
1	$\text{Pu}(\text{NO}_3)_4$	afferent total	1.1945×10^{-3}	1.9175×10^{-3}	2.0867×10^{-3}	2.6135×10^{-3}	1.9031×10^{-3}	3.5448×10^{-3}	3.9001×10^{-3}	4.2709×10^{-3}
2	$\text{Pu}(\text{NO}_3)_4$	efferent acellular	3.8234×10^{-5}	9.1568×10^{-5}	2.1897×10^{-5}	4.6999×10^{-5}	2.7986×10^{-5}	3.3679×10^{-4}	3.4806×10^{-4}	2.4410×10^{-4}
2	$\text{Pu}(\text{NO}_3)_4$	efferent cellular	2.7371×10^{-7}	2.0095×10^{-6}	1.6083×10^{-6}	1.8426×10^{-6}	1.2751×10^{-6}	4.6563×10^{-6}	5.8712×10^{-6}	8.6243×10^{-6}
2	$\text{Pu}(\text{NO}_3)_4$	efferent total	3.8508×10^{-5}	9.3577×10^{-5}	2.3505×10^{-5}	4.8842×10^{-5}	2.9261×10^{-5}	3.4145×10^{-4}	3.5393×10^{-4}	2.5272×10^{-4}
3	polydispersed PuO_2	afferent acellular	1.3006×10^{-4}	3.9877×10^{-5}	2.2003×10^{-5}	3.4751×10^{-5}	2.292×10^{-5}	1.6848×10^{-5}	8.3249×10^{-6}	4.9622×10^{-6}
3	polydispersed PuO_2	afferent cellular	3.1870×10^{-5}	1.2720×10^{-5}	5.6784×10^{-6}	6.5376×10^{-6}	7.9090×10^{-6}	1.1130×10^{-5}	1.4647×10^{-5}	1.9601×10^{-5}
3	polydispersed PuO_2	afferent total	1.6193×10^{-4}	5.2597×10^{-5}	2.7681×10^{-5}	4.1289×10^{-5}	2.8201×10^{-5}	2.7978×10^{-5}	2.2972×10^{-5}	2.4564×10^{-5}
4	polydispersed PuO_2	efferent acellular	2.0684×10^{-6}	9.0957×10^{-6}	3.1347×10^{-6}	3.5650×10^{-6}	2.0138×10^{-6}	1.0461×10^{-6}	6.0329×10^{-7}	2.6483×10^{-7}
4	polydispersed PuO_2	efferent cellular	9.4752×10^{-8}	3.1475×10^{-7}	4.8416×10^{-7}	6.7608×10^{-7}	7.4834×10^{-7}	1.1097×10^{-6}	3.4623×10^{-6}	4.2572×10^{-6}

Table 13 (continued)

Dog No.	Plutonium Characteristics	Lymph Characteristics	Elapsed Hours After Implant							
			0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
4	polydispersed PuO ₂	efferent total	2.1631x10 ⁶	9.4105x10 ⁶	3.6149x10 ⁶	4.2411x10 ⁶	2.7621x10 ⁶	2.1558x10 ⁶	4.0656x10 ⁶	4.5220x10 ⁶
5	0.16μ PuO ₂	afferent acellular	7.3152x10 ⁵	3.3995x10 ⁵	7.2019x10 ⁵	2.7196x10 ⁵	6.4527x10 ⁵	8.2721x10 ⁵	6.3457x10 ⁵	4.5830x10 ⁵
5	0.16μ PuO ₂	afferent cellular	4.1993x10 ⁴	1.2515x10 ⁴	2.1738x10 ⁴	1.1130x10 ⁴	1.0579x10 ⁴	1.3679x10 ⁴	1.1823x10 ⁴	6.8021x10 ⁵
5	0.16μ PuO ₂	afferent total	4.9308x10 ⁴	1.5946x10 ⁴	2.8949x10 ⁴	1.3850x10 ⁴	1.7032x10 ⁴	2.1952x10 ⁴	1.8168x10 ⁴	1.1385x10 ⁴
6	0.16μ PuO ₂	efferent acellular	1.3221x10 ⁶	4.7467x10 ⁵	4.4067x10 ⁶	BMDA*	BMDA	BMDA	BMDA	BMDA
6	0.16μ PuO ₂	efferent cellular	5.0678x10 ⁶	1.0828x10 ⁵	3.9031x10 ⁶	3.1477x10 ⁶	3.9346x10 ⁷	1.3692x10 ⁶	4.9419x10 ⁶	8.4987x10 ⁷
6	0.16μ PuO ₂	efferent total	6.3899x10 ⁶	5.8295x10 ⁵	8.3098x10 ⁶	3.1477x10 ⁷	3.9346x10 ⁷	1.3692x10 ⁶	4.9419x10 ⁶	8.4987x10 ⁷
7	0.54μ PuO ₂	afferent acellular	2.0368x10 ⁵	5.5596x10 ⁵	7.9844x10 ⁵	7.2189x10 ⁵	5.0400x10 ⁵	4.4339x10 ⁵	7.7108x10 ⁵	5.5692x10 ⁵
7	0.54μ PuO ₂	afferent cellular	3.3475x10 ⁵	8.0242x10 ⁵	1.0068x10 ⁴	4.2312x10 ⁵	7.8164x10 ⁵	7.1583x10 ⁵	1.5813x10 ⁴	9.0249x10 ⁵
7	0.54μ PuO ₂	afferent total	5.3825x10 ⁵	1.3584x10 ⁴	1.8524x10 ⁴	1.1450x10 ⁴	1.2861x10 ⁴	1.1159x10 ⁴	2.3524x10 ⁴	1.4594x10 ⁴

Table 13 (continued)

Dog No.	Plutonium Characteristics	Lymph. Characteristics	Elapsed Hours After Implant							
			0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
8	0.54μ PuO ₂	efferent acellular	1.2990x10 ⁶	9.3527x10 ⁷	1.2470x10 ⁶	BMDA	BMDA	BMDA	BMDA	BMDA
8	0.54μ PuO ₂	efferent cellular	4.7631x10 ⁸	2.1658x10 ⁸	4.3312x10 ⁹	2.1650x10 ⁸	4.3302x10 ⁸	3.0743x10 ⁷	7.7942x10 ⁸	8.6628x10 ⁹
8	0.54μ PuO ₂	efferent total	1.3466x10 ⁶	9.5692x10 ⁷	1.2513x10 ⁶	2.1650x10 ⁸	4.3302x10 ⁸	3.0743x10 ⁷	7.7942x10 ⁸	8.6628x10 ⁹
9	1.05μ PuO ₂	afferent acellular	6.2678x10 ⁵	4.5459x10 ⁵	1.4051x10 ⁴	7.2320x10 ⁵	BMDA	BMDA	BMDA	BMDA
9	1.05μ PuO ₂	afferent cellular	1.8459x10 ⁴	9.6513x10 ⁵	2.0921x10 ⁵	4.7353x10 ⁶	9.4705x10 ⁶	4.9935x10 ⁶	1.2053x10 ⁶	1.2398x10 ⁴
9	1.05μ PuO ₂	afferent total	2.4727x10 ⁴	1.4197x10 ⁴	1.6143x10 ⁴	7.7055x10 ⁵	9.4705x10 ⁶	4.9935x10 ⁶	1.2053x10 ⁶	1.2398x10 ⁴
10	1.05μ PuO ₂	efferent acellular	BMDA	BMDA	BMDA	BMDA	BMDA	BMDA	BMDA	BMDA
10	1.05μ PuO ₂	efferent cellular	BMDA	BMDA	BMDA	2.7551x10 ⁶	3.0994x10 ⁶	1.8941x10 ⁶	1.2914x10 ⁶	8.6101x10 ⁸
10	1.05μ PuO ₂	efferent total	BMDA	BMDA	BMDA	2.7551x10 ⁶	3.0994x10 ⁶	1.8941x10 ⁶	1.2914x10 ⁶	8.6101x10 ⁸

* Below minimal detectable activity.

Figure 18. Fraction of initial implant dose of $\text{Pu}(\text{NO}_3)_4$ in afferent lymph.

X = acellular fraction

Square = cellular fraction

Figure 19. Fraction of initial implant dose of $\text{Pu}(\text{NO}_3)_4$
in efferent lymph.

X = acellular fraction

Square = cellular fraction

afferent and efferent lymph enables one to determine how the lymph node functions in removing plutonium from lymph in the early hours after a wound exposure.

Soluble plutonium can be considered to be in an ionic form when it enters the body. Two different reactions may take place when entry occurs via a wound -- polymeric or colloidal forms of plutonium may result from hydrolysis or it can be complexed by proteins and other types of biological macromolecules (Taylor, 1973). The first reaction is predominant. Thus, most of the plutonium becomes fixed at the wound site initially. Over a period of a year, up to 70% of a soluble form of plutonium, such as $\text{Pu}(\text{NO}_3)_4$, may be removed from a wound site. This removal occurs either by phagocytosis of Pu particles or by dissolution (Bistline, 1973).

It was found that the acellular Pu fraction for the $\text{Pu}(\text{NO}_3)_4$ dogs was ten times higher in afferent lymph than in efferent lymph. This fact indicates that either Pu was moving into the vascular system via lymph-venous communications or that it was being picked up and retained by cells within the lymph node. From the data reported by Bistline (1973) it is known that high activity is found in the lymph node within the first few hours following $\text{Pu}(\text{NO}_3)_4$ implants. This would point to the retention of plutonium within the lymph node although it does not preclude the movement of some plutonium into the vascular system via lymph-venous communications.

The cellular Pu fraction was consistently less in efferent lymph although the difference was slight. This would indicate that plutonium was being retained by cells in the lymph node and was not passing through the node into efferent lymph channels.

Insoluble plutonium was handled differently by the body. When entering the body, insoluble Pu was in a particulate form and therefore, the large excesses of ionic Pu did not exist. With time, phagocytosis or dissolution of the Pu particles takes place, where upon the same processes that moved soluble Pu occurred.

The data obtained for the dogs implanted with polydispersed PuO_2 is presented in Figures 20 and 21. In both afferent and efferent lymph Pu concentration in the acellular fraction was found to be 5 to 10 times higher than in the cellular fraction until three hours post-implant when the positions reversed. The initially higher concentration of Pu in acellular lymph may be due to a particle surface phenomenon whereby dissolution initially occurs at a much greater rate on the outermost surface of the particle. This assumption would explain the initial peak in ionic Pu available for movement by the acellular fraction that was seen (Raabe, 1974).

The plutonium concentration in the acellular fraction of afferent lymph was found to be 50 times higher than in the acellular fraction of efferent lymph. On the other hand, the cellular fraction in afferent lymph was a 1000 times higher than the cellular fraction of efferent

Figure 20. Fraction of initial implant dose of polydispersed
 PuO_2 in afferent lymph.

X = acellular fraction

Square = cellular fraction

Figure 21. Fraction of initial implant dose of polydispersed
 PuO_2 in efferent lymph.

X = acellular fraction

Square = cellular fraction

lymph at 0.5 hour post-implant but was reduced to a factor of 10 at the end of 4.0 hours. Both of these findings indicate that a large amount of the PuO_2 is being retained in the popliteal lymph node. These findings parallel the data obtained by Bistline (1973) for PuO_2 . Dagle (1973) showed that PuO_2 particles were being phagocytosed by macrophages within the lymph node. This mechanism would account for the retention of PuO_2 in the lymph node.

Overall, it can be stated that for dogs implanted with $\text{Pu}(\text{NO}_3)_4$, the fraction of the initial implant dose was found to be higher by two to three orders of magnitude in the acellular fraction of both afferent and efferent lymph than in the respective cellular fractions. This contrasts with the lymph of dogs implanted with polydispersed PuO_2 where the acellular fraction of initial implant dose is only slightly higher until 3.0 hours post-implant, when the trend reverses itself. The higher concentration of Pu in the acellular fractions of lymph from dogs implanted with $\text{Pu}(\text{NO}_3)_4$ gives further evidence for the theory that most of the initial plutonium transport of soluble forms of plutonium is via a protein bound mechanism.

The fraction of the initial implant dose in afferent lymph (acellular and cellular fractions) in two different dogs, one implanted with $\text{Pu}(\text{NO}_3)_4$ and the other with polydispersed PuO_2 , is plotted in Figure 22. The data shows that the initial implant dose moving in afferent lymph was approximately two orders of magnitude higher in

—

Figure 22. Fraction of initial implant dose in afferent lymph
(acellular and cellular).

X = $\text{Pu}(\text{NO}_3)_4$

Square = polydispersed PuO_2

the dog implanted with $\text{Pu}(\text{NO}_3)_4$ than in dog implanted with polydispersed PuO_2 during the 4.0 hour collection period. This indicates that the soluble $\text{Pu}(\text{NO}_3)_4$ was leaving the wound site much faster than the insoluble PuO_2 which is in agreement with the conclusions reached by Bistline (1973).

The fractions of the initial implant dose in efferent lymph (acellular and cellular fractions) in two different dogs, one implanted with $\text{Pu}(\text{NO}_3)_4$ and the other with polydispersed PuO_2 , are presented in Figure 23. The fraction of the initial implant dose moving in efferent lymph was found to be approximately ten times higher in the dog implanted with $\text{Pu}(\text{NO}_3)_4$ during the 4.0 hour collection period implying that a greater amount of the particular PuO_2 was retained within the popliteal lymph node. This is to be expected since particulate material is more easily retained in lymph nodes (Yoeffy and Courtice, 1970).

2. Monodispersed plutonium oxide particles

Particle size effects have often been used as an explanation for the protracted movement of polydispersed PuO_2 from a wound site (Dagle, 1973; Gomez, 1973; Bistline, 1973). Three sizes (0.16μ , 0.54μ , and 1.05μ) of monodispersed plutonium oxide particles were used to investigate the particle size problem in the lymphatic transport of PuO_2 .

Figure 23. Fraction of initial implant dose in efferent lymph (acellular and cellular).

X = $\text{Pu}(\text{NO}_3)_4$

Square = polydispersed PuO_2

This experiment compared the transport of 0.16μ monodispersed PuO_2 particles in afferent and efferent lymph. The cellular fraction is consistently higher in Pu content in afferent lymph but varies for efferent lymph (Figures 24 and 25). The lower plutonium content found in efferent lymph results from the retention of PuO_2 particles in the lymph node. This observation was further emphasized by the fact that the plutonium concentration was 100 times higher in afferent lymph than efferent lymph. No data for the acellular fraction in efferent lymph is reported after 1.5 hours post-implant because it was below minimal detectable activity (0.4 pico curies).

The fraction of monodispersed (0.54μ) PuO_2 in afferent and efferent lymph is plotted in Figures 26 and 27. For the afferent lymph the values obtained were about the same throughout the 4.0 hour collection period. No data for the efferent acellular fraction beyond 1.5 hours is plotted in Figure 27 because it was below minimal detectable activity. The cellular fraction of efferent lymph contained three orders of magnitude less plutonium than afferent lymph. This again would indicate that substantial amounts of PuO_2 particles are being retained within the lymph node.

The amount of monodispersed (1.05μ) PuO_2 in the acellular fraction of afferent lymph was found to drop below minimal detectable activity after 2.0 hours (Figure 28). The plutonium content in the cellular fraction of afferent lymph decreased steadily with time except

Figure 24. Fraction of initial implant dose of monodispersed
(0.16 μ) PuO₂ in afferent lymph.

X = acellular fraction

Square = cellular fraction

Figure 25. Fraction of initial implant dose of monodispersed
(0.16 μ) PuO₂ in efferent lymph.

X = acellular fraction
Square = cellular fraction

Figure 26. Fraction of initial implant dose of monodispersed
(0.54μ) PuO_2 in afferent lymph.

X = acellular fraction

Square = cellular fraction

Figure 27. Fraction of initial implant dose of monodispersed
(0.54 μ) PuO₂ in efferent lymph.

X = acellular fraction

Square = cellular fraction

b

P

Figure 28. Fraction of initial implant dose of monodispersed
(1.05 μ) PuO₂ in afferent lymph.

X = acellular fraction

Square = cellular fraction

for the point 4.0 hours post-implant (Figure 28). This would suggest that either the body's ability to remove these large PuO_2 particles diminishes with time or that smaller PuO_2 particles were present in the monodispersed PuO_2 which could also account for early high transport of PuO_2 . The efferent cellular data for monodispersed (1.05μ) PuO_2 indicates that there was a delay period before the PuO_2 particles started to move out of the popliteal lymph node (Figure 29). Whether this was due to the release of the large particles from radiation damaged cells is not known.

Overall, the data obtained for all three particle sizes demonstrates that the cellular fraction of lymph, whether afferent or efferent, transports a greater amount of Pu than the acellular fraction. It was also found that the range of particle sizes studied in these experiments indicates that the phagocytic cells in lymph can transport varying particle sizes of PuO_2 ; thus, particle size does not prohibit cellular transport of PuO_2 in particles up to 1.0μ .

A composite graph of all three particle sizes (0.16μ , 0.54μ , and 1.05μ) of PuO_2 in total afferent lymph (cellular and acellular fractions) is presented in Figure 30. As can be observed, it is difficult to discern differences in the transport of the specific particle sizes, although the trend suggests that the larger particles are transported more slowly.

Figure 29. Fraction of initial implant dose of monodispersed (1.05 μ) PuO₂ in efferent lymph. Square = cellular fraction. No acellular data is presented because all samples were below minimal detectable activity.

Figure 30. Fraction of initial implant dose in afferent lymph
(acellular and cellular).

X = monodispersed (0.16 μ) PuO₂

Square = monodispersed (0.54 μ) PuO₂

Diamond = monodispersed (1.05 μ) PuO₂

In Figure 31, the data obtained for the same three particle sizes of PuO_2 were plotted to compare differences in total efferent lymph. However, no definite particle size differences can be distinguished.

Data in Figure 30 suggests that particle size, below 1.0μ , is not the significant factor in the total lymph transport of PuO_2 from a wound site that other researchers have suggested (Johnson, 1969; Gomez, 1973; Bistline, 1973). This assumption was based on the fact that the various sizes of PuO_2 particles were undergoing dissolution at the wound site and were being transported in the acellular fraction in the first few hours following implant (see section IV this chapter). This data represents, however, only ten individual dogs; thus, precluding extensive statistical inference.

Information obtained for all particle sizes indicated that the lymph node is an effective filtering organ for PuO_2 particles of varying sizes transported in lymph. It is significant that smaller Pu particles (0.16μ) are moving beyond the lymph node via the cellular fraction of efferent lymph after only 0.5 hours but that the larger particles (1.05μ) do not pass through until 2.0 hours post-implant. Therefore, for plutonium actually transported in particulate form, it can be concluded the lymph node is much more effective in stopping the larger particles. This observation implies that the size of the particle reaching the lymph node will influence whether the particle is retained in the lymph node or passes through it.

Figure 31. Fraction of initial implant dose in efferent lymph
(acellular and cellular).

X = monodispersed (0.16 μ) PuO₂

Square = monodispersed (0.54 μ) PuO₂

Diamond = monodispersed (1.05 μ) PuO₂

B. Discontinuous Gel Electrophoresis

Discontinuous gel electrophoresis was used to assess whether there was a qualitative change in the protein binding of plutonium in lymph as it passes through a lymph node and to evaluate any possible effect that the chemical form of Pu may have on the protein binding mechanism. Discontinuous gel electrophoresis has already been used as an effective tool in elucidating which proteins are binding plutonium (see section II. D. this chapter). Experiments were conducted to compare plutonium binding in afferent and efferent lymph using two different chemical forms of plutonium, $\text{Pu}(\text{NO}_3)_4$ and poly-dispersed PuO_2 .

Plutonium concentrations contained in sections of disc gels used to separate $\text{Pu}(\text{NO}_3)_4$ contaminated afferent and efferent lymph proteins are plotted in Figures 32 and 33. Major peaks in sections 1-3, sections 8-12, and sections 32-37 are seen in Figure 32. The first peak in Figure 32 corresponds to a precipitation site where the Pu contaminated lymph protein was applied to the gel. Previous studies with standardized lymph proteins indicated that the second peak corresponds to the location of the protein transferrin, while the third peak corresponds to the location of albumin and part of the ion front. The height of the first two peaks is much less (0.07) than the third peak (0.21).

Figure 32. Fraction of Pu detected in sections of discontinuous gels on which $\text{Pu}(\text{NO}_3)_4$ contaminated afferent lymph proteins were electrophoresed.

Figure 33. Fraction of Pu detected in sections of discontinuous gels on which $\text{Pu}(\text{NO}_3)_4$ contaminated efferent lymph proteins were electrophoresed.

Figure 33 shows major peaks in sections 8-11, sections 28-33, and sections 33-35. From previous studies with standardized lymph proteins, it was found that the first peak corresponds to the location of transferrin, the second peak to albumin, and the third to part of the ion front. This means that when plutonium nitrate is introduced into the body through a wound site it travels primarily with the lymph proteins albumin and transferrin. This was observed in both afferent and efferent lymph. Transferrin has been cited as the chief protein binding Pu in serum (Poppewell and Boocock, 1967; Stevens et al., 1968; Turner and Taylor, 1968b; Taylor, 1973). All of these studies involved plutonium incubated in large amounts of serum or following injection of plutonium intravenously; thus, the plutonium was exposed to large amounts of protein which included transferrin. This contrasts to the situation in lymph where protein concentration is much lower and the lymph at the wound site may only amount to one or two ml; thus, the amount of transferrin available for the binding of plutonium molecules is significantly less. Norwood (1972) outlines the problems of comparing plutonium binding when the plutonium is injected intravenously as opposed to the industrial wound case. It is believed that in the latter case the excess plutonium will bind to other proteins such as albumin. In comparing plutonium binding to different proteins, Turner and Taylor (1968b) indicated that a small amount of plutonium may be bound to serum albumin. This means that albumin

could be used as a secondary site for plutonium binding. According to Wasserman and Mayerson (1952) albumin concentration is higher in lymph than in serum. Data presented in this chapter, section III. D., supports this contention. Thus, it is not unlikely to find plutonium bound to albumin in lymph.

Plutonium concentrations in sections of disc gels used to separate polydispersed PuO_2 contaminated afferent and efferent lymph proteins are presented in Figures 34 and 35. Major peaks were found in sections 9-12, sections 30-32, and sections 32-36 (Figure 34). Previous studies with standardized lymph proteins indicated that the first peak corresponds to the location of transferrin and the second corresponds to the location of albumin. The third peak is part of the ion front.

Major peaks in Figure 35 were found in sections 7-9, sections 29-33, and sections 33-36. Previous studies with standardized lymph proteins indicated that the first two peaks corresponded to the location of transferrin with the low value in section 9 due to a sectioning problem. Since the proteins are in bands and sections are cut equidistant, a particular section can incorporate no bands, one band, the edge of a band, or the edges of two bands. If plutonium is associated with one or both bands, sectioning can cause artificially high or low points with these bands. The third peak corresponds to the location of albumin and the fourth to part of the ion front.

Figure 34. Fraction of Pu detected in sections of discontinuous gels on which polydispersed PuO_2 contaminated afferent lymph proteins were electrophoresed.

Figure 35. Fraction of Pu detected in sections of discontinuous gels on which polydispersed PuO_2 contaminated efferent lymph proteins were electrophoresed.

The data from Figures 34 and 35 also suggest that albumin and transferrin are the two primary proteins binding Pu in afferent and efferent lymph when a dog is implanted with polydispersed PuO_2 . This differs from the data of Gomez (1973) who reported that 54% of the plutonium moved with transferrin. As discussed previously, Gomez did not properly identify the location of transferrin or describe the effect of electrophoresis on the plutonium peaks. The meaning of the 54% is therefore uncertain. In addition, he did not report albumin as playing a significant role in Pu binding to lymph proteins. This is because the albumin was never separated out properly from the other globulins when the gels were sectioned.

C. Isoelectric Focusing Gel Electrophoresis

To get a comparison with the results of the discontinuous gel system, isoelectric focusing gel electrophoresis was performed on the same lymph samples. The isoelectric focusing system has been characterized as a useful technique in clarifying the plutonium-lymph protein relationship (see section II. D. this chapter).

Plutonium concentrations in sections of isoelectric focusing gels on which electrophoresis was carried out to separate $\text{Pu}(\text{NO}_3)_4$ contaminated afferent and efferent lymph proteins were plotted (Figures 36 and 37). Major peaks in sections 1-3, sections 7-10, and sections 18-24 are seen in Figure 36. The first peak corresponds to precipitation at the application site of the Pu-contaminated afferent lymph.

Figure 36. Fraction of Pu detected in sections of isoelectric focusing gels on which $\text{Pu}(\text{NO}_3)_4$ contaminated afferent lymph proteins were electrophoresed.

Figure 37. Fraction of Pu detected in sections of isoelectric focusing gels on which $\text{Pu}(\text{NO}_3)_4$ contaminated efferent lymph proteins were separated.

Standardized lymph protein studies indicated that peak two corresponds to albumin while peak three is transferrin.

Figure 37 contains major peaks in sections 7-10 and sections 19-25. Sections 19-25 were lumped together because section 23 was low due to the slicing problem. Again peak one corresponds to albumin and peak two to transferrin.

Combining the information derived from Figures 36 and 37, it can be concluded that, in afferent and efferent lymph collected from $\text{Pu}(\text{NO}_3)_4$ implanted dogs, albumin and transferrin are the two principal proteins binding plutonium. This confirmed the results obtained with discontinuous gel electrophoretic techniques.

Fraction of Pu in each section of isoelectric focusing gels, used to separate polydispersed PuO_2 contaminated afferent and efferent lymph proteins, were plotted (Figures 38 and 39).

Major peaks occurred in sections 8-11 and sections 19-23 (Figure 38). Sections 19-23 were lumped together because section 21 was low in activity due to the sectioning problem. Comparison with standardized lymph proteins indicated that peak one corresponds to albumin, while peak two is transferrin.

The data plotted in Figure 39 contains major peaks in sections 7-11 and sections 16-23. Sections 16-23 were lumped together because section 19 was low due to the slicing problem again. Previous

Figure 38. Fraction of Pu detected in sections of isoelectric focusing gels on which polydispersed PuO_2 contaminated afferent lymph proteins were separated.

Figure 39. Fraction of Pu detected in sections of isoelectric focusing gels on which polydispersed PuO_2 contaminated efferent lymph proteins were separated.

standardized lymph protein studies indicated that peak one corresponded to albumin and peak two to transferrin.

Taken together, Figures 38 and 39 confirmed the results obtained on polydispersed PuO_2 movement in afferent and efferent lymph proteins determined with discontinuous gel electrophoresis. Both electrophoretic techniques indicated that plutonium, soluble or insoluble, is handled the same way in afferent and efferent lymph; thus, no change in the binding of plutonium takes place after it has moved through a lymph node.

D. Cellulose Acetate

To compare distribution of particular protein groups (albumin, α -globulins, β -globulins, and γ -globulins) in Pu contaminated afferent and efferent lymph with time, electrophoresis was performed with cellulose acetate as the support medium. The data for these comparisons are presented in Tables 14 and 15. The means for the different time periods (control pre-implant, 1.0 hour post-implant, and 4.0 hours post-implant) were compared and found not to be significantly different. This indicates that plutonium does not elicit a qualitative change in the lymph proteins during the early hours following implant. The lack of a shift in protein groups indicates that plutonium was constantly being picked up from the wound site and transported by the same lymph protein groups.

Table 14

Cellulose Acetate Separation of Afferent Lymph
Proteins Contaminated with Plutonium

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
<u>Control Pre-implant</u>					
1	$\text{Pu}(\text{NO}_3)_4$	73%	6%	16%	6%
3	polydispersed PuO_2	57%	11%	18%	14%
5	0.16 μ PuO_2	55%	13%	20%	12%
7	0.54 μ PuO_2	55%	12%	16%	16%
9	1.05 μ PuO_2	62%	9%	19%	9%
		<u>60.4$\pm$7.6%</u>	<u>10.2$\pm$2.8%</u>	<u>17.8$\pm$1.8%</u>	<u>11.4$\pm$4.0%</u>
<u>1.0 Hour Post-implant</u>					
1	$\text{Pu}(\text{NO}_3)_4$	56%	13%	18%	13%

Table 14 (continued)

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
3	polydispersed PuO_2	61%	7%	17%	14%
5	0.16 μ PuO_2	59%	12%	11%	18%
7	0.54 μ PuO_2	66%	6%	19%	9%
9	1.05 μ PuO_2	54%	11%	24%	11%
		<u>59.2$\pm$4.7%</u>	<u>9.8$\pm$3.1%</u>	<u>17.8$\pm$4.7%</u>	<u>13.0$\pm$3.4%</u>
<u>4.0 Hours Post-implant</u>					
1	$\text{Pu}(\text{NO}_3)_4$	61%	8%	18%	13%
3	polydispersed PuO_2	48%	9%	17%	26%
5	0.16 μ PuO_2	54%	12%	16%	18%

Table 14 (continued)

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
7	0.54 μ PuO ₂	57%	7%	18%	18%
9	1.05 μ PuO ₂	75%	7%	11%	7%
		59.0 $\pm$ 10.2%	8.6 $\pm$ 2.1%	16.0 $\pm$ 2.9%	16.4 $\pm$ 7.0%

Table 15

Cellulose Acetate Separation of Efferent Lymph
Proteins Contaminated with Plutonium

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
<u>Control Pre-implant</u>					
2	$\text{Pu}(\text{NO}_3)_4$	59%	11%	16%	13%
4	polydispersed PuO_2	66%	3%	26%	5%
6	0.16 μ PuO_2	46%	9%	20%	26%
8	0.54 μ PuO_2	52%	19%	15%	14%
10	1.05 μ PuO_2	63%	10%	17%	10%
		<u>57.2$\pm$8.2%</u>	<u>10.4$\pm$5.8%</u>	<u>18.8$\pm$</u>	<u>13.6$\pm$7.8%</u>
<u>1.0 Hour Post-implant</u>					
2	$\text{Pu}(\text{NO}_3)_4$	48%	15%	18%	18%

Table 15 (continued)

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
4	polydispersed PuO_2	70%	5%	18%	7%
6	0.16 μ PuO_2	67%	5%	17%	10%
8	0.54 μ PuO_2	54%	6%	17%	22%
10	1.05 μ PuO_2	56%	14%	16%	14%
		<u>59.0$\pm$9.2%</u>	<u>9.0$\pm$5.0%</u>	<u>17.2$\pm$0.8%</u>	<u>14.2$\pm$6.0%</u>
<u>4.0 Hours Post-implant</u>					
2	$\text{Pu}(\text{NO}_3)_4$	59%	11%	19%	11%
4	polydispersed PuO_2	56%	9%	21%	15%
6	0.16 μ PuO_2	58%	4%	22%	16%

Table 15 (continued)

Dog No.	Plutonium implanted	Protein Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
8	0.54 μ PuO ₂	45%	10%	27%	17%
10	1.05 μ PuO ₂	54%	10%	15%	18%
		54.4 $\pm$ 5.6%	8.8 $\pm$ 2.8%	20.8 $\pm$ 4.4%	14.8 $\pm$ 4.4%

Since cellulose acetate strip electrophoresis has often been used as a technique in grossly determining the protein group which binds serum plutonium (Turner and Taylor, 1968a), an experiment comparing cellulose acetate electrophoresis with the two gel techniques (discontinuous and isoelectric focusing) was of interest. Afferent and efferent lymph proteins, contaminated with $\text{Pu}(\text{NO}_3)_4$ or polydispersed PuO_2 , were again electrophoresed.

The fraction of Pu associated with each protein group, when electrophoresed on cellulose acetate strips, is presented in Table 16. It was found that the percentage of Pu was highest in β -globulins followed by albumin, γ -globulins and α -globulins. This was true for both afferent and efferent lymph proteins contaminated with either soluble or insoluble forms of plutonium. One of the principal proteins in β -globulins is transferrin (Feeney and Allison, 1968). From this experiment it can be concluded that albumin and transferrin are the principal lymph proteins carrying plutonium. This agrees with the results obtained with discontinuous and isoelectric focusing gel electrophoresis. Turner and Taylor (1968a) observed that 70-80% of the plutonium was associated with β_1 -globulin fraction of horse serum. This was much less than the values seen in lymph, but the problems with comparing serum and lymph binding of plutonium have already been discussed.

Table 16

Percentage of Plutonium in Specific Protein Groups
of Lymph Separated on Cellulose Acetate

Dog No.	Plutonium implanted	Plutonium Concentration			
		Albumin	α -globulins	β -globulins	γ -globulins
<u>Afferent Lymph</u>					
1	$\text{Pu}(\text{NO}_3)_4$	28%	12%	41%	19%
3	polydispersed PuO_2	34%	13%	37%	16%
<u>Efferent Lymph</u>					
1	$\text{Pu}(\text{NO}_3)_4$	36%	6%	47%	11%
3	polydispersed PuO_2	21%	13%	53%	13%

E. Immunoelectrophoresis

Since immunoelectrophoresis has been used to study the binding of small molecules to proteins, it was felt that this technique might be of value in investigating the plutonium-lymph relationship.

Typical immunoelectrophoresis slides obtained for afferent and efferent lymph are pictured in Figures 40 and 41. Albumin and transferrin bands are indicated in both figures. The immunoelectrophoresis slides obtained compare favorably with those seen by Roberts and Courtice (1969).

Autoradiography of the immunoelectrophoresis slides failed to produce any concentrations of alpha tracks along the protein arcs. In fact, alpha tracks were scattered quite randomly throughout the immunoelectrophoresis slides. Several factors could account for this failure to demonstrate the alpha tracks in the transferrin and albumin protein arcs as expected. It is possible that the plutonium could have dissociated because of the high ma used during the electrophoretic process. It is also possible that an agar-plutonium interaction could have occurred whereby the plutonium became bound by the agar. The antibody could have interacted with the plutonium. Most likely, however, the problem resided in the time necessary for diffusion (24-48 hours) so that the antigen-antibody interaction could occur. This long diffusion process could have allowed the weak plutonium-protein bands to break and create a situation favorable to the influx of other ions

Figure 40. Immunoelectrophoresis slide of canine afferent lymph.

A = albumin

T = transferrin

Figure 41. Immunoelectrophoresis slide of canine efferent lymph.

A = albumin

T = transferrin

found on the slide that could bind plutonium. These results lead to the conclusion that immunoelectrophoresis is not a good technique for studying the plutonium-lymph relationship, at least under the conditions set forth in this experiment.

IV. Fission Track Analysis

Research conducted at Rocky Flats Plant (Dow Chemical Company, Denver, Colorado) indicated that fission track analysis was a good technique for measuring particle size of PuO_2 (Hayden, 1970). Since lymph cells can be collected on a filter, it was thought that fission track analysis might be used to determine the size of PuO_2 particles that they were transporting. Monodispersed PuO_2 particles were used for implantation so that a comparison could be made with the particles in the lymph cells.

It was found that in afferent lymph all three particle sizes implanted were slightly reduced in size (Table 17). This observation would point to dissolution or selective transport of smaller particles, since the particles were not purely monodispersed as indicated by the standard deviations in Table 4. It is believed that dissolution was at least partially responsible for the size reduction as indicated by the plutonium content in the acellular fraction of monodispersed PuO_2 implanted dogs in Table 13. Because of the low activity in the efferent lymph of the dogs implanted with 0.54μ and 1.05μ monodispersed PuO_2 , only two and three particles were detected on the filters respectively;

Table 17

Plutonium Oxide Particles Sizes (μ) in Lymph Cells

Plutonium Implanted	Lymph Vessel Collected	Sample Time (Hours) Post-implant	Particle Size in Cells	Fraction of Particles of Given Size
Monodispersed PuO_2 0.16 μ	afferent	3.5	0.15 μ	65%
Monodispersed PuO_2 0.16 μ	efferent	2.5	0.11 μ	60%
Monodispersed PuO_2 0.54 μ	afferent	3.0	0.45 μ	72%
Monodispersed PuO_2 0.54 μ	efferent	3.5	0.45 μ	100%
Monodispersed PuO_2 1.05 μ	afferent	3.0	0.82 μ	63%
Monodispersed PuO_2 1.05 μ	efferent	1.0	0.52 μ	60%

thus, it is difficult to ascertain whether the trend showing an even greater reduction in the particle sizes in efferent lymph was true. If this trend is in fact correct, then the lymph node is selectively removing larger particles. Gomez (1973) reported that only small alpha stars (no size given) were seen in afferent lymph on the first day of implant. This contrasts with the fission track data in that all three sizes of PuO_2 particles were seen in efferent lymph.

The particle size information showed that particles in afferent lymph are smaller than at the implant site and that after passing through the lymph node the average is reduced even further.

This was consistently seen with all three particle sizes.

CHAPTER V

SUMMARY

These experiments were undertaken to study the uptake and transport mechanism of soluble ($\text{Pu}(\text{NO}_3)_4$) and insoluble (polydispersed PuO_2) plutonium in afferent and efferent lymph from the popliteal lymph node of dogs after simulated wound exposures.

Normal values for some of the constituents of afferent and efferent lymph were determined. The normal cell count was 220 cells/ mm^3 in afferent lymph and 7398 cells/ mm^3 in efferent lymph. Protein concentration in afferent lymph was 13.81 ± 4.02 mg/ml, while efferent lymph contained 21.43 ± 2.80 mg/ml.

The protein concentration in afferent and efferent lymph did not change during the 4.0 hour period, post-implant, that lymph samples were collected. Qualitative changes in specific lymph protein groups (albumin, α -globulins, β -globulins, and γ -globulins) were not observed during the 4.0 hour collection period.

The validity of using electrophoretic techniques in studying the Pu-lymph relationship was substantiated. Discontinuous and isoelectric focusing gel electrophoretic systems were useful in determining which proteins bind Pu, but were inaccurate in determining

percentages of Pu binding to specific proteins because Pu atoms were being stripped off of the proteins during electrophoresis.

Lymph was partitioned into acellular and cellular fractions. Plutonium nitrate concentration was higher in the acellular fraction of both afferent and efferent lymph than in the cellular fraction, while the polydispersed PuO_2 concentration was higher in the cellular fraction.

Monodispersed PuO_2 particles (0.16μ , 0.54μ , and 1.05μ) were compared on the basis of their movement in lymph from a simulated wound exposure. Particle size was not found to be an important factor in the total movement (acellular and cellular lymph fractions) of Pu in afferent and efferent lymph. Regardless of particle size, more Pu moved in the cellular fraction of both afferent and efferent lymph than in the acellular fraction.

Discontinuous gel, isoelectric focusing gel, and cellulose acetate strip electrophoresis indicated that transferrin and albumin were the two principal proteins transporting plutonium in the acellular fraction of both afferent and efferent lymph.

Immunoelectrophoresis proved to be an inaccurate technique to study protein-bound plutonium.

Fission track analysis indicated that dissolution of the monodispersed Pu particles takes place at the wound site. Plutonium oxide particles at the implant site were larger than in afferent lymph and

larger in afferent lymph than in efferent lymph. The dissolution of PuO_2 accounts for its rapid movement in acellular lymph following implant.

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