THE UNIVERSITY OF MANITOBA

IMMUNOLOGICAL ANALYSIS OF PARTICULATE ANTIGEN(S) OF HUMAN LEUKEMIA

BY

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INTRODUCTION

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Human leukemia, first recognized in 1847, is a neoplastic change in the blood-forming tissues, characterized by an uncontrolled proliferation of leucocytes and their precusors with invasive tendencies.

Whilst many types of animal leucosis have been demonstrated to be of viral origin, the etiology of human leukemia is not yet established. Although Herpes-type viruses and C-type particles have been found repeatedly in the blood plasma and other materials derived from human leukemia, the immunological properties of these particles have not been investigated.

It has been the object of the present research work to extract particulate antigens from human leukemic organs and examine the purified extract for immunological specificity and cross-reactivity with antigens of other animal leukemias.

In designing this research project, the hypothetical agent of human leukemia was considered to be a subcellular entity such as a virus or a non-structural virus-like unit, a slow latent virus or a macro-molecular complex, capable of initiating the leukomogenic process.

It was hoped that elucidation of the immunological properties of this agent would contribute towards the understanding of the complex etiology of human leukemia.

REVIEW OF THE LITERATURE

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The etiology of malignant neoplasms has been the subject of an enormous amount of research since the beginning of the century.

1. HISTORY OF EXPERIMENTAL VIRAL ONCOLOGY

In 1908, Ellerman and Bang were able to transmit myeloblastosis and erythroblastosis by means of cell-free filtrates, thus establishing the viral etiology of avian leukosis. But the idea that an infectious agent could be the cause of malignant neoplasms did not come to be recognized until Rous (1911) discovered that avian sarcomas were also caused by a virus. Rous sarcoma virus is a C-type RNA virus, the first of many such viruses now implicated in all forms of leukosis, including human leukemia. Bittner (1936) made the next great advance when he discovered the slow acting mouse mammary tumour agent. This B-type RNA virus provided an important model for human carcinomas, in later research work. The 1950s were exciting times in experimental viral oncology because of the development of tissue culture and numerous technical advances, amongst which was the tolerant host technique. L. Gross (1951), using immune tolerant mice, was able to demonstrate a leukomogenic virus in the blood and tissues of mice of two high incidence leukemic strains. The Gross virus is also a slow acting virus and is recoverable from infected mice even in the absence of evert disease.

Since then, many similar murine leukemia viruses have been discovered and characterized, and in all cases they were observed to be the classical C-type RNA virus. However, these viruses were all from inbred populations representing special cases irrelevant to the normal population. But Jarrett et al (1964) showed that lymphosarcoma and leukemia of normal domestic cats

were induced by viruses, also of the classical C-type RNA variety.

II. EVIDENCE FOR VIRUS-LIKE PARTICLES ASSOCIATED WITH HUMAN LEUKEMIA

The first suggestive evidence for a viral agent associated with human leukemia came from the work of Dmochowski (1957), who reported virus-like particles in the ultra-thin sections of biopsy tissues from a patient with acute lymphatic leukemia. Similar findings were reported by Ito (1958) and Beard (1958). Thus began an intensive research for virus-like particles in human leukemic specimens using electron microscopy.

The evidence for C-type particles was confirmed by Murphy and Furtado (1963), Almeida et al (1963), Burger et al (1964) and Dalton et al (1964). Porter et al (1964) found C-type particles in 8 out of 56 patients with acute leukemia. Negroni (1964) also reported the isolation of C-type viruses from 10 to 25 bone marrows from patients with acute leukemia. These viruses were maintained in serial passage and were capable of inducing cytopathic changes in infected cells. It was named the Negroni virus. Unfortunately Grist and Fallon (1964) subsequently reported isolation of mycoplasma from Negroni's cultures, but this still did not rule out the possibility of co-infection. As a matter of fact Murphy et al (1965) found 10 of 58 specimens from leukemic children contained mycoplasma. Hayflick and Koprowski (1965) described agar isolation of mycoplasma from one of three children with acute lymphoblastic leukemia. Mycoplasma orale was isolated by Anderson and Barile (1966) from 4 of 10 patients with acute leukemia. All controls were negative.

Benyesh-Melnick (1966), over a four-year period, demonstrated a correlation between electron microscopy findings and tissue culture results with matieral obtained from children with leukemia and infectious mono-nucleosis. She found a great similarity between these findings and those

of animal leukemias, especially avian leukosis. She examined, by negative

staining in electron microscopy, the plasma or serum of both groups and

found particles similar to the Avian Myeloblastosis Viruses in 67% of the

leukemic children, 75% of children with infectious mononucleosis and 3% of

normal healthy children of the same age. She was able to obtain lymphoblastoid

transformation in fibroblast cultures and the supernatent fluids from trans-

formed cultures yielded virus-like particles. But attempts to demonstrate a

possible infectious role for the viruses associated with the two diseases failed

because of the inability to serially passage the virus.

Levine et al (1967) were able to correlate the presence of C-type particles,

resembling murine leukemia viruses in size and shape, found in plasma pellets

of leukemic patients, to their clinical status. Acute patients had more particle

than chronic patients, while non-leukemic patients and leukemic patients in

remission showed no particles at all.

While Dmochowski and his team at Houston continued to find C-type particles

in direct examination of human leukemia, Grace and others at Roswell Park grew

Herpes-type viruses (HTV) in their human leukemia cultures from bone marrows

and leucocytes (Iwakata and Grace, 1964; Grace, 1967). Their results were

confirmed by Moore et al (1966), Zeve et al (1966) and de Harven (1967).

Pope (1968) established two cell lines from peripheral leucocytes of

Australian leukemic patients. Electron microscopy revealed HTV distinct

from Herpesvirus hominis in infectivity. Ito (1969) was able to detect HTV

chromosomal markers in two human cell lines derived from embryonic cultures

exposed to human leukemic culture fluid in vitro. Chandra et al (1971)

detected an unusual cytoplasmic envelopment of HTV in a human leukocyte

culture. Kinderklin (1971) compared the HTV he obtained from the bone marrow

of a leukemic infant to Herpes Simplex virus and found similarity in morphology

and stages of maturation to Herpes virus, but a slight difference in size and mean dry mass.

The evidence for HTV was further strengthened by findings of HTV in Burkitt's Lymphoma (Epstein et al, 1964; Griffin et al, 1966; O'Connor et al, 1965). It is now well established that this virus, termed the Epstein-Barr Virus (EBV) is implicated in infectious mononucleosis, Burkitt's Lymphoma and nasopharyngeal carcinoma.

Other types of virus particles have also been found. Smith et al (1964) reported Myxovirus-like particles associated with human leukemia, while Rausing (1970) was able to detect by negative staining in electron microscopy evidence for a papova virus of the polyoma type in a chronic lymphatic leukemia patient. However, it is most likely that these are opportunistic infections, as was the case with mycoplasma.

Contrary evidence for the presence of a virus associated with leukemia was revealed by Bernard and Leplus (1964) in their fine structure studies of normal and malignant human lymph nodes. Arnoult and Haguenau (1966), in their study of blood plasma, cerebral spinal fluid, and megakaryocytes from bone marrow, also failed to find any particles. These studies were supported by evidence from Uzman et al (1966) and Clarkson et al (1967), the latter using continuous suspension cultures of seven new cell lines established from the blood of acute leukemic patients.

Although Murphy and Zarafonetis (1968) failed to find particles in leucocyte cultures of leukemic patients, they did observe virus-like particles in leukemic spleens and other organs. Rechar (1969) examined suspension cultures derived from human leukemic and non-leukemic sources. Electron microscopy revealed virus-like particles for Hodgkin's culture from bone marrow and spleen; lymphosarcoma from bone marrow; carcinoma of cervix and myeloprolifera-tive disease from spleen. The results were negative for bone marrow and buffy coat culture of myelomonocytic leukemia and lymph node of lymphosarcoma.

The most recent development occured at Houston, where Priori et al (1971) announced the presence of C-type particles in a cell line (ESP-1) established from a child who had Burkitt's lymphoma. This coupled with Temin's reverse transcriptase enzyme discovery created quite a lot of excitement in the field of cancer research. However, Gilden (1971) later reported that ESP-1 actually contained a mouse antigen which implied that the virus was a contaminant. So the mystery of C-type virus of human origin remained unsolved and the race for finding a causal agent for human cancer continues.

III. TRANSMISSION OF HUMAN LEUKEMIA TO ANIMALS

Indirect evidence for a virus as a causal agent of human leukemia came from experiments on the transmission of human leukemia to various kinds of animals.

In 1957, Riman and Vesely transplanted human leukemia into rats. In that same year, Schwartz, Schoolman and others successfully induced leukemia in 326 adult AKR mice using filtrates of brains of patients who died of leukemia. The control group did not develop any signs of leukemia. These brain filtrates were also passed serially through Swiss mice five times and then injected into 40 AKR mice. Thirty-eight of these developed leukosis indicating replicating activity in these filtrates.

Parnes and Suntsova (1959) also managed to induce leukemia in 4 of 46 mice injected with human leukemic spleen extracts. Dmochowski et al (1959) used cell-free extracts of lymph nodes, brain, spleen, and kidneys of persons who died of leukemia to induce leucosis in newborn Swiss mice. Ten to twenty percent developed leukemia as compared to one to three percent in uninoculated controls. The organs of infected mice revealed

virus-like particles similar to those seen in human leukemic tissue.

Similar findings were reported by Delong (1960) and Ageenko (1961). Bergolz (1960), in experiments involving 6000 animals, was able to show very convincingly that human leukemic tissue contained a cell-free, heat-labile factor, capable of producing leukemia in 37% of the mice a few months after injection of this "active principle" subcutaneously or directly into the spleen and thymus. The principle was extractable by salt and water-alcohol mixtures from tissue homogenates, nuclei and cytoplasmic granules of human leukemic tissue. However, Benyesh-Melnick (1966) was unable to demonstrate any transformation in tissue cultures derived from human embryonic haematopoetic organs or bone marrows of normal children, using plasma or sera containing high concentrations of virus-like particles, supernatent of spontaneously transformed bone marrow, or transformed cells, sonically disupted or frozen and thawed. But in the following year, Osato and Ito (1969) reported transformation in vitro of human embryo fibroblast tissues using leucocyte culture fluids or human leukemic serum. So it appears that a filterable agent of some kind is associated with human leukemia.

IV. IMMUNOLOGICAL STUDIES AS A TOOL FOR THE ELUCIDATION OF VIRAL ETIOLOGY OF HUMAN LEUKEMIA.

Immunological studies have been greatly enhanced by the discovery of two new techniques - immunofluorescence and immune tolerance.

De Carvalho (1960) segregated leukemic specific antigens from human leukemia and tumoral cells by fluorocarbon extraction. More convincing evidence came from Garb et al (1962) who injected leukemic buffy coats into immune tolerant rabbits (neonatal rabbits injected with normal blood constituents to induce tolerance and therefore would selectively produce

antibodies to leukemic materials). Specificity of the leukemic antigen was demonstrated by Ouchterlony immunodiffusion and skin test by Arthus reaction. Greenspan et al (1963) demonstrated a leukemia specific tissue antigen by passive cutaneous anaphylaxis and also showed cross-reactions between the antigens of mice and man. Izawa et al (1966) not only confirmed the existence of the leukemic specific antigen in man and animals by fluorescent antibody, leukoagglutination and immunodiffusion, but went on to investigate therapeutic uses of its antiserum in man. A decrease in leucocyte count and in percentage of abnormal cells was induced but proved only temporary. Sakurai (1966) confirmed the above results and also showed, by I^{131} - labelled antisera against chronic myelogenous leukemia, that this antisera conjugated with pathological cells six hours after it was given to a patient.

Fink and Cowles (1968) reviewed the use of immunological techniques in the study of human leukemia. They concluded that although there was a good correlation between immunofluorescent cells in the buffy coat and C-type viral particles in plasma, immunofluorescence was the more sensitive technique. This was confirmed by Yohn and Grace (1967) at Roswell Park. Fink, using membrane immunofluorescence, found cross-reactions of the C-type particles and its antisera with Burkitt's lymphoma cells, herpesvirus and its antisera, and to anti-Rauscher serum. So the question of C-type particles and HTV in human leukemia became more complex. It is quite possible that both co-exist in the leukemic cell but how each manifests itself remains a puzzle.

Viza et al (1970) extracted and solubilized a leukemia specific antigen from leucocytes of human leukemic patients, and produced antisera to a partially purified preparation of this antigen. The possibility of using

this antigen and its antisera for diagnostic and therapeutic purposes
lends a great deal of importance to this approach in human leukemia research.

V. THE ENZYME CONTROVERSY

A clue to the possible viral etiology of human leukemia seemed to
have been uncovered in 1970 when Baltimore (1970) and Temin and Mizuntani
(1970) detected RNA-directed DNA polymerase activity in virions of RNA
tumour viruses. Spiegelman et al (1970) and Temin's group also found
double-stranded nucleic acid dependent DNA polymerase activity and DNA
endonucleases in these viruses. (Mizutani, 1971).

Then Gallo (1970) found the same enzymes in human leukemic cells, so
it appeared very possible that the enzyme was present in these cells
because of a causal RNA virus. However, further work has shown that the
two enzymes were different and that the ones found in leukemic cells were
also found in rapidly growing normal cells such as embryonic cells. But
in spite of these later findings, the importance of the enzyme should not
be overlooked. Obviously much more work has to be done to associate the
enzyme with a causal virus. Watanabe and Haruna (1969) found self-
replicating RNA in polio-infected Hela cells, Echovirus infected FL cells,
Friend leukemic cells in mice and human acute myeloid leukemic cells. This
also seems to point to the presence of viral RNA being a causative agent of
human leukemia. So the enzymes may well be coded for in normal cells and
only produced when rapid replication is called for. It stands to reason
that the virus when attached to the host genome could simply tap this
convenient resource of enzymes in the cells for its own replication.

VI. EVIDENCE FOR VIRUS-LIKE PARTICLES IN OTHER MAMMALIANS

Avian leukosis viruses and murine leukemia viruses have been well

characterized and need not be presented here, but evidence for virus-like

particles in other mammals may substantiate the rationale for finding

virus-like particles in human leukemia. So a brief summary of the evidence

is presented below.

Rats:- The evidence for virus-like particles in leukemic rats appears scarce

and inconclusive. Weinstein and Moloney (1965) found C-type particles 70 nm

in diameter in rat chloroleukemia. However several groups of workers were

able to induce leukemia in rats using mouse leukemia virus and subsequently

observed a large number of typical mouse leukemia virus particles in receptive

rats. Dmochowski et al (1962, 1964) first reported this observation which

was confirmed in 1963 by Okano et al (1966). Feldman and Gross (1966)

further observed that the particles multiplied in the megakaryocytes of the

rat.

Hamsters:- Bernard and Tournier (1964) were the first to identify C-type

particles in tumours of hamsters. They observed these particles in 7 of

11 cases and considered it to be a widespread latent infection. In 1966,

Steinback et al confirmed the above findings in a variety of hamster neoplasms.

Guinea Pigs:- Opler (1967) described viral particles found in transplantable

guinea pig lymphoblastic leukemia by electron microscopy and infectivity

studies. They were found in ultrathin sections of lymphatic tissue,

megakaryocytes and plasma pellets.

Cattle:- Particles similar to murine leukemia virus had been observed in

cattle (bovine leukosis) in the thymus, lymph nodes, spleen and mammary

glands. The transmission of bovine leukosis is therefore believed to be

vertical (diaplacentary or milk) and/or horizontal (cutaneous or contact).

Dogs:- Chapman et al (1967) described virus-like particles in canine

leukemia. The particles from the spleen and lymph nodes did not appear to be virus-like particles but normal "coated vesicles". However pellets from canine cell cultures revealed characteristic C-type particles. Rickard and Post (1968) were able to transmit canine mast cell leukemia by cell-free filtrates into two week puppies, providing further evidence for viral etiology of canine leukemia.

Cats:- Jarrett et al (1964) first observed particles morphologically similar to those occuring in murine and avian leukemia in cytoplasmic vesicles of transplanted cat lymphosarcoma, which is the most frequently encountered manifestation of the leukemia complex in the cat. The disease was transmitted by cell-free filtrates and virus particles were found in the platelets and megakaryocytes of kittens twenty-eight days after infection. Jarrett (1969) also replicated the feline leukemia virus in cells of man, dogs and pigs. Geering (1968) found that feline leukemia viruses shared one of the group-specific antigens of the murine leukemia virus, gs-3. It was confirmed by Sarma in both immunodiffusion and complement-fixation studies. This inter-species immunological reaction reflects some degree of homology between the leukemia viruses isolated from different animals.(Sarma,1971)

MATERIALS   AND   METHODS

MATERIALS AND METHODS

I. SOURCE OF MATERIALS

Livers and spleens from autopsies of persons who died of leukemia
were obtained for the extraction of particulate antigens. These specimens
were extracted as soon as available and when possible were frozen at -20° C
until the time of extraction. A full autopsy report was obtained with each
specimen. Special attention was given to the age, sex and type of leukemia
diagnosed.

Control specimens of livers and spleens were obtained from non-leukemic
non-infectious deaths such as heart failures, multiple traumatic injuries,
coronary thromboses, and drug overdose. As far as possible, these were
matched with the leukemic group for age, sex and blood type. (see Tables I
and II)

II. EXTRACTION OF PARTICULATE ANTIGENS

Particulate antigens were prepared from livers and spleens by the
modified method of Greenspan et al (1964) and Kwapinski (1969).

Each specimen was minced and washed in 0.01 M phosphate-buffered saline
(PBS, see Appendix I) with centrifugation at 5000g for 15 minutes until no
traces of blood were visible. The packed volume and weight of the tissue
fragments were then determined. The washed material was suspended as
15-20% (w/v) in 0.01 M PBS, pH 7.2, and homogenized for 10 minutes in a
Sorvall electric omnimixer at 4° C. The homogenate was re-adjusted to
pH 7.2 and hyaluronidase was added to a final concentration of 250 units
per 100 ml of homogenate. The mixture was left at room temperature for
one hour with slow stirring. The digestate was centrifuged at 6000g for
20 minutes. The sediment was collected and resuspended in 0.01M PBS
containing 1% formalin and left at room temperature for 3-5 hours and at

TABLE I

LEUKEMIC SPECIMENS

No.	AUTOPSY NO.	AGE SEX	DATE OF DEATH	AUTOPSY DIAGNOSIS
L-1	69B371	23/F	15/12/69	Acute myeloid leukemia
L-2	71B47	24/M	24/2/71	Acute Lymphoblastic Leukemia
L-3	A70/45	4/M	7/4/70	Acute myeloblastic leukemia
L-4	70B272	69/M	12/9/70	Acute monomyelocytic leukemia
L-5 *	PM274-68			
L-6	86243	14/M	7/12/70	Promyelocytic leukemia
L-7	71B50	20/M	1/3/71	Acute Lymphoblastic Leukemia
L-8	71B51	35/M	1/3/71	Acute Lymphocytic Leukemia

* Specimen obtained from Dr. E. R. Brown of the Chicago Medical
School. No history was given.

TABLE II

NON-LEUKEMIC SPECIMENS

No.	AUTOPSY NO.	AGE SEX	DATE OF DEATH	AUTOPSY DIAGNOSIS
N-1	A25643	58/M	23/11/70	Multiple Traumatic Injuries
N-2	25335	59/M	25/6/70	Congestive Heart Failure
N-3	70B129	62/F	22/4/70	Congestive Heart Failure
N-4	70B322	52/F	7/11/70	Cardiac Arrest and Lung Carcinoma
N-5	A25597	46/F	2/11/70	Congestive Heart Failure
N-6	25802	5/F	8/2/71	Multiple Traumatic Injuries
N-7	A25905	35/M	31/3/71	Coronary Thrombosis
N-8	25943	28/M	18/4/71	Drug Overdose
N-9	25962	56M	27/4/71	Coronary Thrombosis
N-10	25980	42/M	3/5/71	Coronary Thrombosis
N-11	25999	61/M	12/5/71	Ruptured Aneurysm
N-12	26002	23/F	13/5/71	Car Accident

4° C overnight. The contents of the cells in the formolized suspension was released by sonication at oscillations of 9Kc/sec. for 20 minutes in a Raytheon sonicator. The disrupted material was centrifuged at 4° C at 6000g for 20 minutes. The supernatent was dialysed against several changes of 0.01M PBS overnight to remove excess formalin, while the sediment, consisting of cell debris and possibly undisrupted cells, was resonicated to increase the final yield of particles. The dialysed material was recentrifuged at 20,000g for 20 minutes and clarified by passing through a Millipore 0.45u filter. The filtrate was centrifuged at 107,000g in the Spinco L2 ultracentrifuge (SW 25.1 rotor) for one hour. The pellet was collected and resuspended at a concentration of 1 gm wet weight of original tissue to 1 ml of 0.01 M PBS. This suspension was centrifuged at 150g for 5 minutes to remove any clumps, and the supernatent was collected as the crude particulate antigen preparation. A summary of the extraction procedure is presented in Table III.

This preparation was examined by negative staining using 2% phospho-tungstate acid, pH 6.8, in electron microscopy for the presence of virus-like particles. In addition, it was injected into rabbits for the production of crude antiserum.

III. PURIFICATION OF PARTICULATE ANTIGENS

Continuous particle electrophoresis and isopycnic gradient centrifugation were used in purifying the crude antigen obtained.

A. Continuous Particle Electrophoresis

Continuous particle electrophoresis, designed to give two-dimensional separation of particles up to 100 nm in diameter, was used to purify the antigen preparation on the basis of electrophoretic mobility as well as density. The crude antigen preparation was subjected to 600 v,

TABLE III

EXTRACTION PROCEDURE FOR PARTICULATE ANTIGEN(S)

Human Leukemic/Normal

Autopsy Specimen
(note wt. & vol.)

_____

Homogenization

_____

Hyaluronidase Digestion

Sediment   Supernatent
                                                      (discarded)

Sonication

Sediment   Supernatent
(discarded)

Filtration

Pellet   Supernatent
Resuspend
1 c.c. PBS/gm. tissue

┌─────────────────┐
│ ANTIGEN   │
│ PREPARATION   │
└─────────────────┘

Antiserum   Electron Microscopy
Production
in Rabbits
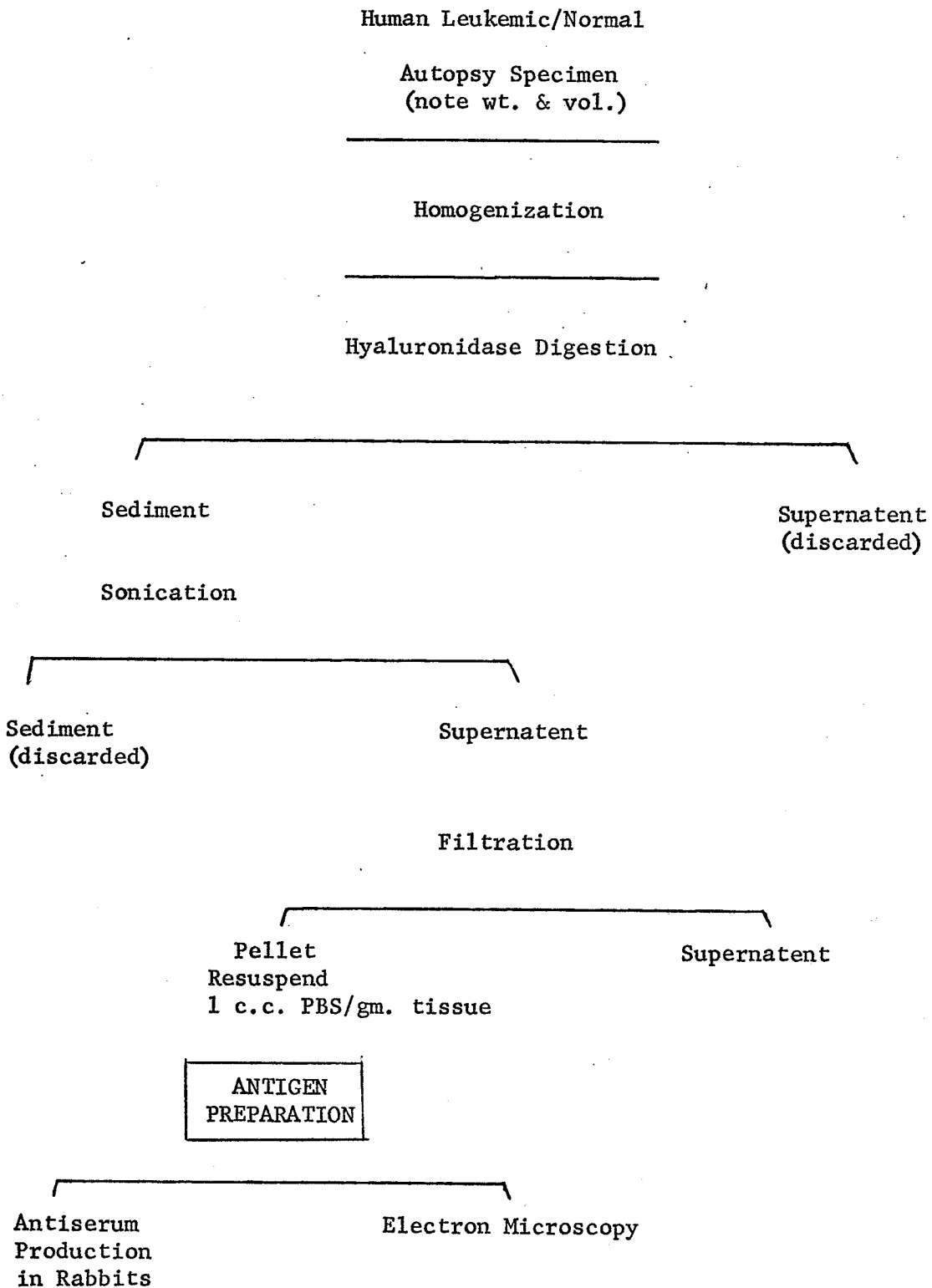
160 mA at 10° C with 0.001 M PBS as curtain buffer and 0.01 M PBS as electrode rinse in a Brinkman's Electrophorectic Separator Model FF-3. Ninety-one tubes were collected and the optical density of each tube was measured at 210 nm, 254 nm and 280 nm wavelength in order to determine contents of peptide linkages, cytosine and aromatic proteins respectively. A curve of the electrophoretic pattern was plotted and the tubes were pooled according to peak areas. These pooled fractions were either dialysed against distilled water overnight and lyophilized or concentrated by ultrafiltration in Diaflo cells using XM-50 membranes, with a molecular weight cut-off of 50,000. Each fraction was brought back to the original sample volume by either method.

Each fraction was then examined in the electron microscope for the presence of virus-like particles by negative staining using 2% phosphotungstate acid adjusted to pH 6.8 with KOH. Particles were enumerated by the method of Watson et al (1963) using latex particles of 88 nm in diameter.

B. Isopycnic Centrifugation

The fractions obtained from continuous particle electrophoresis revealing the presence of virus-like particles were further purified by isopycnic centrifugation in sodium citrate gradients. Gradients were prepared from 15-40% (w/v) sodium citrate in saline pH, 7.0, by layering the different concentrations of sodium citrate into centrifuge tubes and allowing a linear gradient to form overnight. 0.5 ml of the sample was layered onto the top of the gradient and centrifuged at 114,483g in a SW 39 L rotor or SW 65 L titanium rotor for 6 hours. 1 ml fractions were collected and their refractive indices measured in a Bausch & Lomb refractometer. The fractions were dialysed against 0.01 M PBS, pH 7.0, overnight at 4° C and concentrated to one half the original sample volume and re-examined in the

electron microscope for the presence of virus-like particles. Fractions from non-leukemic specimens were similarly processed but reduced to one tenth of the original sample volume to unequivocably ascertain the absence of virus-like particles in these materials.

IV. IMMUNOLOGICAL INVESTIGATION

A. Immunization

Antisera to the partially purified antigen preparations were produced by injection into Albino rabbits weighing 4.0 - 4.5 lbs. according to the immunization schedule presented in Table IV. The crude non-leukemic preparations were also injected as controls.

The rabbits were test bled 10 days after the last injection, and if their sera reacted positively with their homologous antigens by immunodiffusion, they were bled out by cardiac puncture.

B. Treatment of Antisera

Immunoglobulins were extracted from antisera obtained by precipitation using saturated ammonium sulphate. To every 10 ml of serum, 5 ml of saturated ammonium sulphate adjusted to pH 7.8 with 2N NaOH was added in dropwise fashion with constant stirring at 4° C. The suspension was left stirring for an additional 2-3 hours to avoid mechanical trapping of other serum components. It was then centrifuged for 30 minutes at 1400g. The precipate was dissolved in enough saline to restore the volume of solution to that of original serum sample. The fraction was further purified by repeating the precipitation, stirring and re-suspension twice. The last precipitate was dissolved in borate-buffered saline, pH 7.8, (see Appendix II) to a final volume less than one half of the original serum. The precipitate was dialysed against borate-buffered saline for three days at 4° C. The dialysate was changed

TABLE IV

IMMUNIZATION SCHEDULE

FOR

PARTIALLY PURIFIED ANTIGEN (S)

DAY	ROUTE OF INJECTION	DOSE OF ANTIGEN *
1	subcutaneous	0.3 ml
3	subcutaneous	0.3 ml
8	subcutaneous	0.3 ml with 0.3 ml of Freund's incomplete adjuvant
22	intravenous	0.5 ml
26	intravenous	1.0 ml

* All antigen preparations were adjusted to an optical density of 0.7 prior to injection.

daily. The dialysed solution was then centrifuged for 30 minutes at 1400g at 4° C and its protein content estimated by the method of Lowry et al (1951). Leukemic immunoglobulins showing reactions in immunodiffusion with normal antigens were absorbed exhaustively with normal antigen preparations.

C. Serological Tests

1. Immunodiffusion in agarose

Precleaned slides were coated with 4 ml of 0.8% agarose prepared in saline, pH 7.0. Wells were made with Gilman cutter template #71688. Centre wells were filled with the partially purified antigen preparations and peripheral walls were filled with antisera to be reacted.

All leukemic and control preparations were cross-reacted with each other to determine their serological inter-relationships. These slides were incubated in a moist chamber at room temperature for three days. Reactions were read using an oblique light source and magnifying hand lens.

2. Micro-precipitin test in Electron Microscopy

The method of Kelen et al (1971) was adapted for use in the investigation of serological reactivities of the virus-like particles found in the leukemic specimens.

Serial dilutions of the immunoglobulins to be tested, including known positive and negative controls, were made in microtiter plates with U-shaped cups. The antigens were added to each cup. The plates were shaken gently, covered and then placed in 37° C for thirty minutes.

After incubation, the antigen-antibody mixture was removed from the microtiter plate with a Pasteur pipette and a drop was deposited onto the surface of microscope slides coated with 5 ml of 1% Oxoid Ionagar #2 prepared in saline. A 400 mesh carbon-formvar-coated copper grid was placed upside down and floated on the top of this drop. When the fluid

phase of the drop diffused into the agar layer, the grid was peeled off the agar surface with locking tweezers.

These grids were then negatively stained with 2% phospho-tungstate acid, pH 6.8, by putting a drop of stain onto the grid for a few seconds and drawing off the excess by touching the edge of the grid to filter paper. The preparation was screened for presence of antigen-antibody complexes under the electron microscope at a magnification of 30,000.

3. Immunoferritin Studies in Electron Microscopy

a) Source of materials

Partially purified antigen from each leukemic and control specimen was used as sources of antigens.

Immunoglobulins prepared from antisera to the partially purified antigens were used as a source of antibody. In the direct test, antisera to L4S, L4L and a broad spectrum normal antigen preparation (BSN) were conjugated to ferritin. In the indirect test, immunoglobulins were extracted from guinea pig anti-rabbit serum by separation in Sephadex G200 column in Tris buffer (see Appendix III) and used as a source of anti-immunoglobulin.

b) Conjugation

The method of Sri Ram et al (1963) was used. 460 mg of ferritin and 160 mg of immunoglobulins were dissolved in 2% Na_2CO_3 to constitute a 4% protein solution. Protein contents were determined by the method of Lowry (1951). 5 mg of p,p' difluoro-m,m' dinitro diphenyl sulphone (FNPS) in 1 ml acetone was then added to the protein solution, and the whole mixture was stirred at 4° C for 24 hours. It was dialysed against saline pH 7.0.

c) Purification of Conjugates

The dialysed conjugates were then purified by the method of Hsu (1967). The mixture was first centrifuged three times at 100,000 g for $4\frac{1}{2}$ hours. The resulting pellets, consisting of ferritin and ferritin conjugated immunoglobulins, were dissolved in 0.05 M phosphate buffer, pH 7.5, after each run. After the final run, a small quantity of phosphate buffer was added to the pellet, which was then held at 4° C overnight. The solution was passed through a Millipore 0.45u filter and stored at 4° C in sterile tubes until use.

d) The Tests

(i) direct method

The antigens and dilutions of ferritin-conjugated immunoglobulins were reacted at 37° C for thirty minutes and at 4° C overnight in Kahn tubes. The precipitates were then spun down at 9000g and washed in 0.05 M phosphate buffer, pH 7.5, and examined in the electron microscope on a carbon-coated copper grid at a starting magnification of 30,000.

(ii) indirect method

The antigen and unconjugated antibody were reacted as in (i). After the precipitate was washed, it was further incubated with the ferritin labelled guinea pig anti-rabbit serum at 37° C for thirty minutes and at 4° C overnight. The precipitate was washed free of excess antibodies and examined in the electron microscope by putting a drop on a carbon-coated copper grid.

(iii) blocking test

The antigen was reacted with unconjugated homologous antibody and then with its conjugated antibody and again examined in the electron microscope.

A summary of the immunoferritin studies is presented in
Table V.

V. CROSS-REACTION STUDIES

A. Studies with Rous Sarcoma Virus - Rous Associated Virus I, RSV(RAV-I)

RSV(RAV-I) and anti-RSV(RAV-I) were obtained from Dr. A. M. Wallbank
of the Department of Medical Microbiology, University of Manitoba.

A portion of the RSV(RAV-I) was sonicated for thirty minutes to
disrupt the viruses and release the internal antigens. Dilutions of 1:2,
1:4, and 1:8 were made of the anti-RSV(RAV-I) serum in order to react with
the intact and disrupted RSV(RAV-I) in 0.8% agarose slides prepared as
described in Section IV, C., 1.

RSV(RAV-I) and its antisera were also reacted with all the antigens
and antisera of leukemic and non-leukemic specimens.

B. Murine Leukemia Virus Studies

Mice infected with GC and S63 murine leukemia viruses and normal
mice were received from Dr. E. R. Brown of the Department of Microbiology,
Chicago Medical School. They were killed and viruses were extracted from
the spleen and lymph nodes of infected mice by the method of Greenspan et al
(1964) and purified for cross-reaction studies by the method O'Connor et al
(1964). Normal mice were processed the same way as infected mice to serve
as controls.

1. Extraction

The spleen and lymph nodes were dissected from killed mice
and minced in Hanks' balanced salt solution (BSS) containing 1.5 mg %
hyaluronidase to make a 20% (w/v) homogenate. The pH was adjusted to 7.2
with Na HCO_3. The homogenate was digested at room temperature for an hour
with slow stirring. The digestate was then ground in a glass mill to a

TABLE V

<u>IMMUNO-FERRITIN STUDIES</u>

I. Preparation of Conjugated antiserum:-

             Immunoglobulins + ferritin + FNPS

                   $4^{o}C$, 24 hr.

                   dialysis vs saline

                   purification by ultracentrifugation

_____

II. Tests:-

     (i) direct method:

               antigen + ferritin-conjugated antiserum

               $37^{o}c$, 30 min.; $4^{o}C$, 24 hr.

               wash precipitate

_____

     (ii) indirect method:

               antigen + antisera

               $37^{o}C$, 30 min.; $4^{o}C$, 24 hr.

               washed precipitate + ferritin-conjugated guinea
                                      pig anti-rabbit γ-globulin

               $37^{o}C$, 30 min.; $4^{o}C$, 24 hr.

               wash precipitate

_____

III. Electron microscopy by negative staining.

fine suspension. It was then centrifuged at 5° C for 15 minutes at
1500g. The supernatent was passed through a Millipore 0.45u filter,
and then centrifuged in the Spinco Model L Ultracentrifuge (SW25.1 rotor)
for one hour at 107,000g. The pellet containing the virus was resuspended
in cold BSS on the basis of 1 ml per gram weight of wet tissue.

2. Virus·Purification

The virus suspension prepared in 1. was further purified by
isopycnic centrifugation through density gradients of sodium citrate
(15-40%, w/v) using the technique described by O'Connor et al (1964). The
banks produced by centrifugation were isolated and their refractive indices
measured for determination of densities. The fractions were then dialysed
overnight at 4° C against 0.01 M PBS and examined with the electron
microscope by negative staining.

The purified virus suspensions were then disrupted by
sonication and cross-reacted with all other preparations as described in
Section IV, C., 1.

C. Studies with Chronic Leukemic Patients' Plasma

Chronic leukemic patients' plasma were adsorbed with homogenates of
normal human spleens and livers,and blood containing A & B antigens. These
plasmas were then cross-reacted with all other preparations as described in
Section IV, C., 1.

D. Studies with other Sera

Antisera to Rauscher and Moloney viruses and Rickard feline leukemia
viruses were obtained from commercial sources and used undiluted in these
cross-reaction studies. A human serum containing a high titer to Epstein-
Barr virus was also used.

RESULTS

RESULTS

I. PURIFICATION

As the crude antigen preparation contained too much background material for examination of virus-like particles in the electron microscope, it was purified by continuous particle electrophoresis. Figures 1, 2 and 3 represent typical separation profiles of leukemic and non-leukemic antigen preparations. Non-leukemic preparations contained a large central peak (tubes 43-53) and a number of smaller peaks towards the anode. The leukemic preparations, however, showed a larger peak in tubes 24-44 compared to the central peak. This elevation was characteristic of all the leukemic preparations regardless of clinical forms of leukemia. One could infer that this material with a slight negative charge might well be associated with human leukemia.

On examination by electron microscopy, virus-like particles of approximately 80 to 100 nm in diameter were seen in this elevated fraction in leukemic samples. No other fractions in the leukemic or control specimens showed any particles. The particles were doughnut shaped and non-enveloped. (Figure 4)

II. ISOPYCNIC CENTRIFUGATION IN SODIUM CITRATE GRADIENTS.

The pooled fractions containing virus-like particles were further purified by isopycnic centrifugation in sodium citrate gradients (15 - 40%, w/v). One ml fractions were then collected. The refractive index for each was determined, and after dialysis they were examined in the electron microscope. Figure 5 shows the distribution of virus-like particles in the gradient. Most of the particles banded at a buoyant density of 1.18 g/c.c. in sodium citrate but some were as low as 1.16 g/c.c. and some as high as 1.195 g/c.c. These might have represented different

Figure 1. Separation profile of L-1 in continuous
           particle electrophoresis. Curves represent
           optical densities as determined at 210 nm
           (———— ————),254 nm (— — — —) and
           280 nm (- - - - -) wavelengths.

Figure 2. Separation profile of L-3 in continuous
           particle electrophoresis. Curves represent
           optical densities as determined at 210 nm
           (——— ———),254 nm (— — — —) and
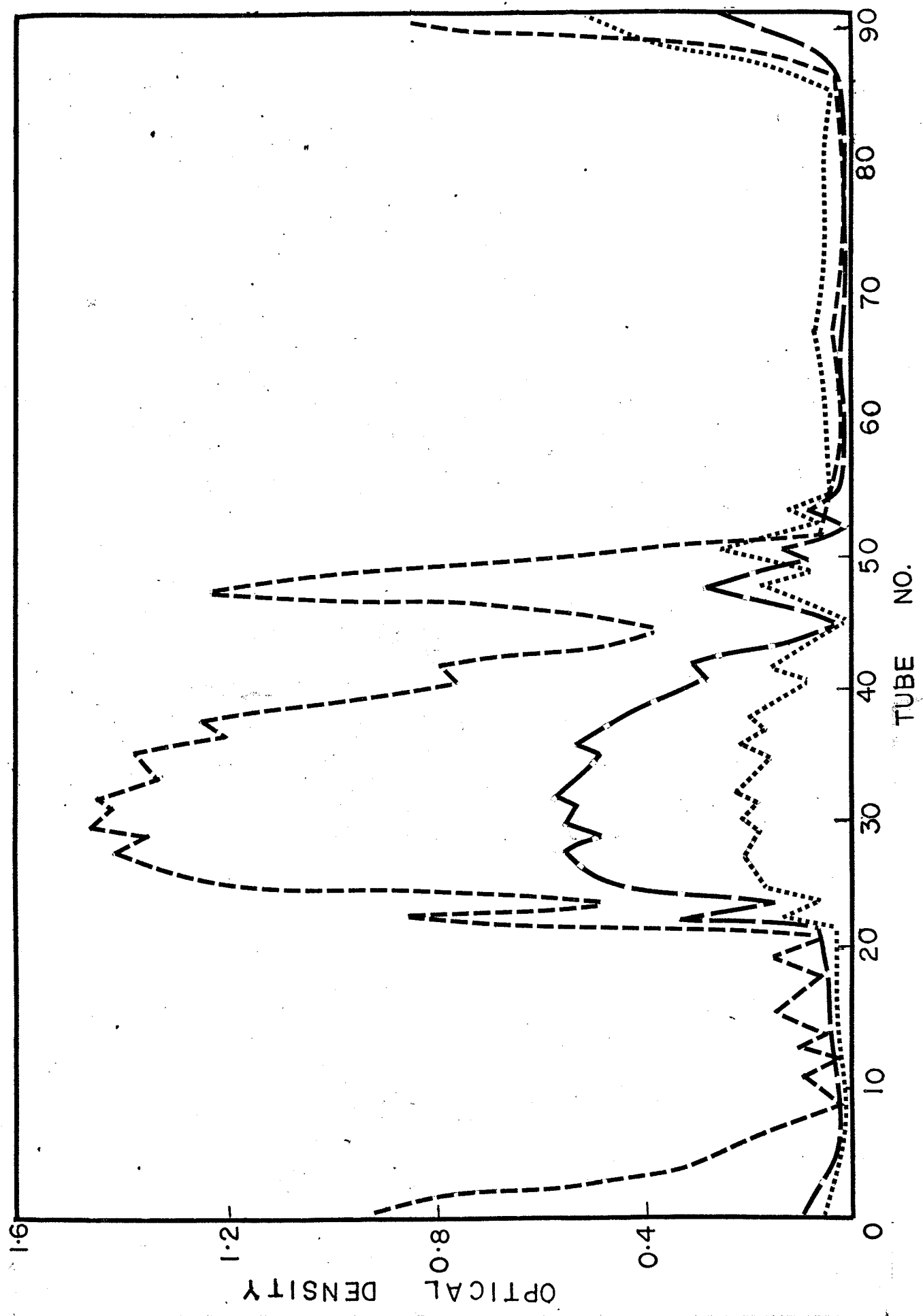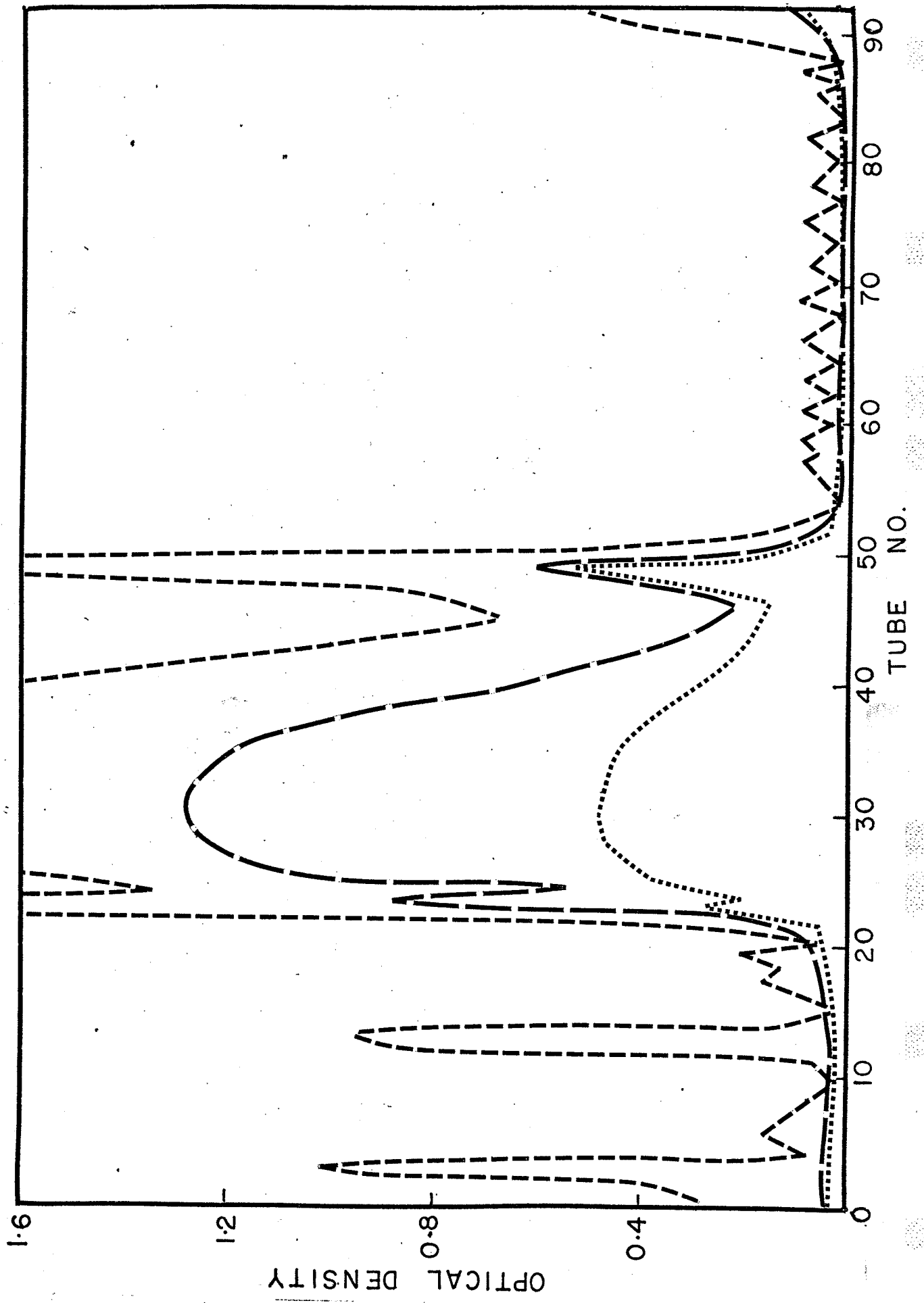   280 nm (- - - - -) wavelengths.

Figure 3. Separation profile of N-4 liver in
continuous particle electrophoresis.
Curves represent optical densities as
determined at 210 nm (————·———) ,254 nm
(— — — —) and 280nm (.........) wavelengths.

OPTICAL DENSITY

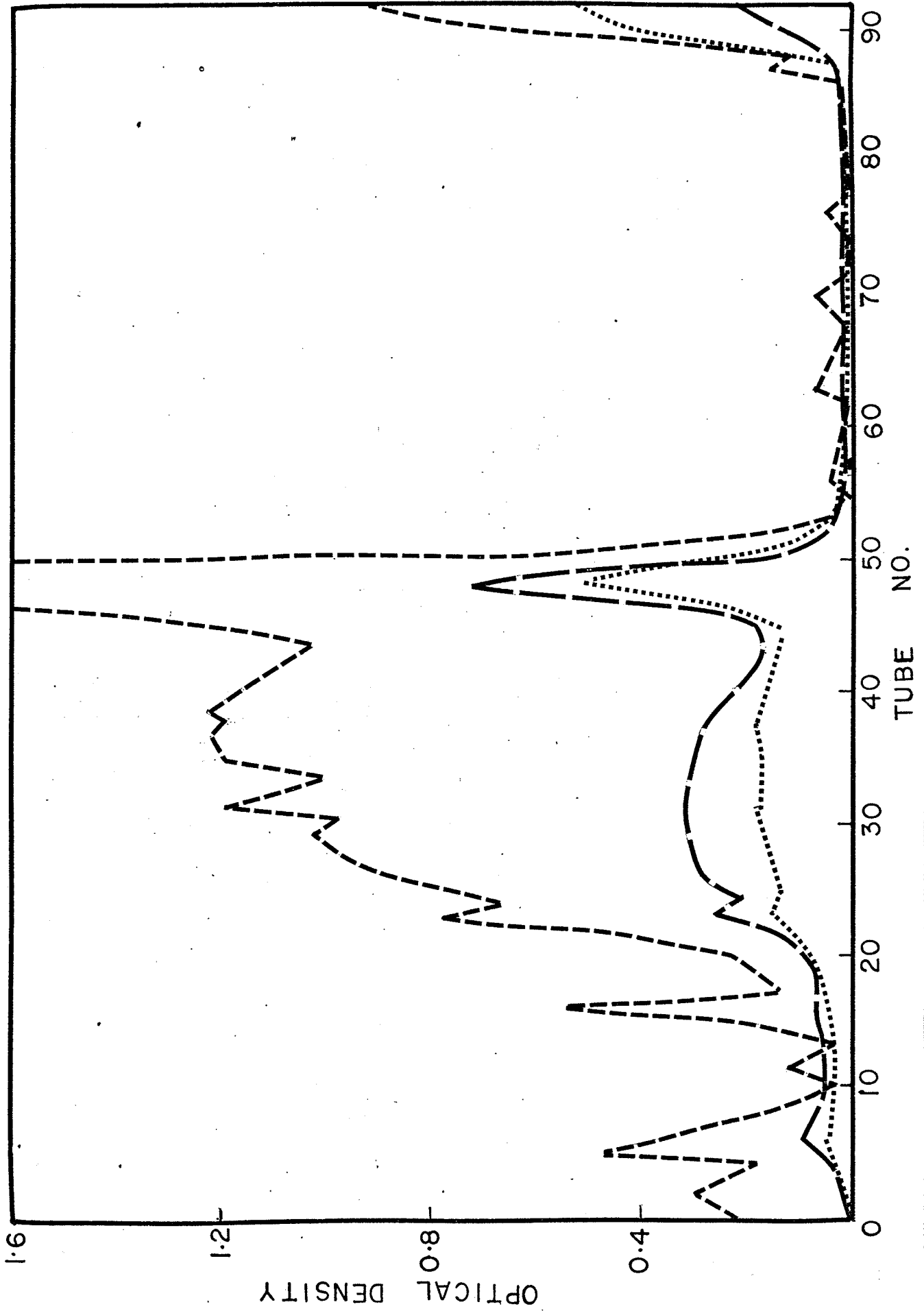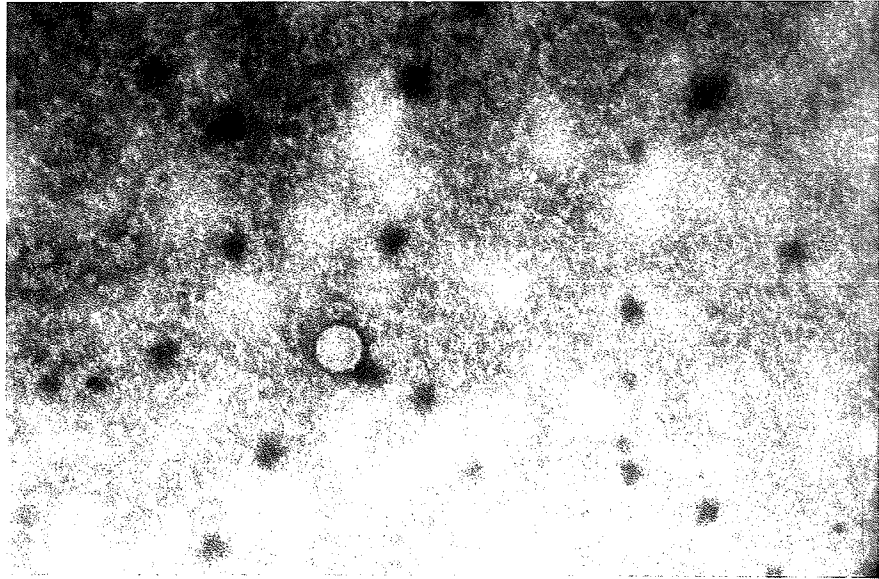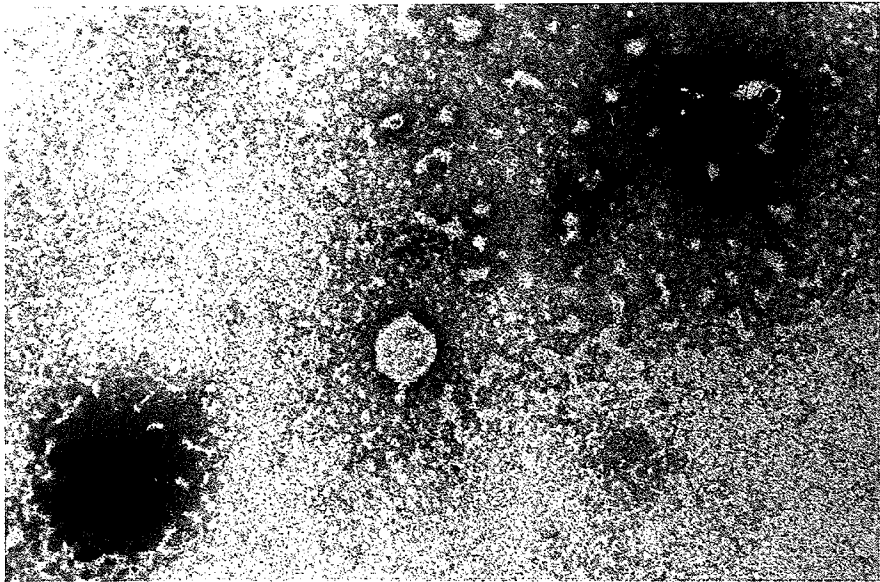TUBE NO.

Figure 4. Virus-like particles found in partially
          purified antigen preparations from L-3.
          " a " : 60,480x,particle size 90 nm.
          " b " : 62,150x,particle size 100 nm.

"a"

"b"

degrees of integrity of the particles or possible association with cell contaminants.

Particle counts were made by examining 20 fields and taking an average of the number of particles seen. Table VI shows the number of particles seen in each specimen. Particle counts were expressed as virus particles per field (400 mesh copper grid) where one particle per field equals approximately 1×10^8 particles/ml of purified preparation.

III. IMMUNOLOGICAL STUDIES.

A. Immunodiffusion Studies

Immunodiffusion studies are summarized in Table VII. The non-leukemic controls did not react with the leukemic preparations while the leukemic preparations exhibited a heterogeneity of cross-reactions. L1 seemed to be relatively unreactive although it contains about 5×10^8 particles/ml. However, it was a small sample and there was not enough to inject into rabbits for antisera production. So studies with L1 were quite limited. The same was true of L3 and L5.

L6 spleen was unusual in that it only reacted with its homologous antisera. The particle count for this sample was very low, so this antigen may not even be associated with leukemia. L6 liver, L7 liver and spleen, and L8 liver appeared to be very good antigens and there were sufficiently high particle counts correlated with the immunodiffusion results.

Figures 6 and 6a show the lines of identity obtained with the various leukemic preparations.

B. Microprecipitin Test in Electron Microscopy

Repeated attempts to visualize aggregation of particles by

Figure 5. Distribution of virus-like particles in
           sodium citrate gradient. ▓▓▓ represents
           area of high particle concentration .
           ▨▨▨▨ represents area of lower particle
           concentration.
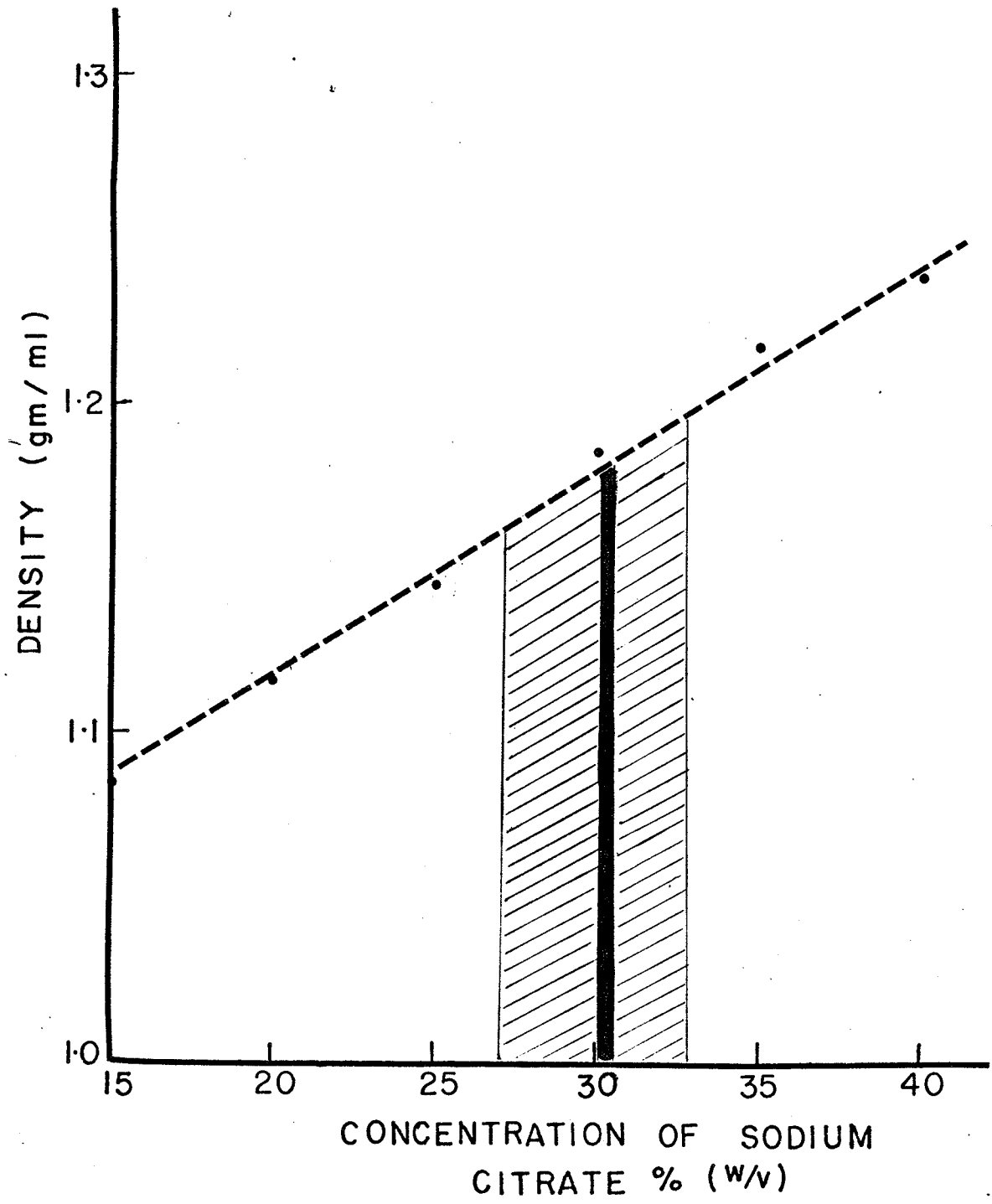
TABLE VI

NUMBER OF VIRUS-LIKE PARTICLES
IN
LEUKEMIC AND NON-LEUKEMIC ANTIGEN PREPARATIONS

	NUMBER OF PARTICLES	
SPECIMEN	LIVER	SPLEEN
L1	n.a.	++
L2	±	±
L3	+++	+++
L4	++	++
L5	n.a.	±
L6	+	±
L7	+++	+++
L8	++	n.a.
Normal	-	-

Legend:

n.a.   = not available

±   = occasional particle seen

+   = 0-5 particles per field

++   = 5-10 particles per field

+++   = > 10 particles per field

-   = no particles.

TABLE VII

IMMUNODIFFUSION REACTIONS (+) DETECTED BETWEEN PARTICULATE ANTIGENS OF HUMAN LEUKEMIA AND ANTISERA AGAINST THE PARTICULATE ANTIGENS

Particulate Antigen Preparation From:	Antiserum against particulate antigen of:									
	L2		L4		L6		L7		L8	"Normal" organs
	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	Spleen	Liver	
L1 Liver	−	−	−	−	+	−	+	+	+	−
L2 Liver	+	+	−	−	+	−	+	+	+	−
L2 Spleen	+	+	+	+	+	−	+	+	+	−
L3 Liver	−	−	−	−	+	−	+	+	+	−
L4 Liver	−	+	+	+	+	−	+	+	+	−
L4 Spleen	−	+	+	+	+	−	+	+	+	−
L5 Liver	−	−	−	+	+	−	+	+	+	−
L6 Liver	+	+	+	+	±	±	+	+	±	−
L6 Spleen	−	−	−	−	+	+	−	−	−	−
L7 Liver	+	+	+	+	+	−	+	+	+	−
L7 Spleen	+	+	+	+	+	−	+	+	+	−
L8 Liver	+	+	+	+	+	−	+	+	+	−
"Normal" Liver & Spleen	−	−	−	−	−	−	−	−	−	+

Figure 6. Immunodiffusion studies of leukemic and
non-leukemic specimens.
"a" : partially purified antigen from L-7
spleen(central well); antisera to L-7
liver,L-8 liver,L-7 spleen,L-6 liver,
L-6 spleen and pooled non-leukemic extract.
(peripheral wells)
"b" : partially purified antigen from L-6
spleen (central well); antisera as in "a".
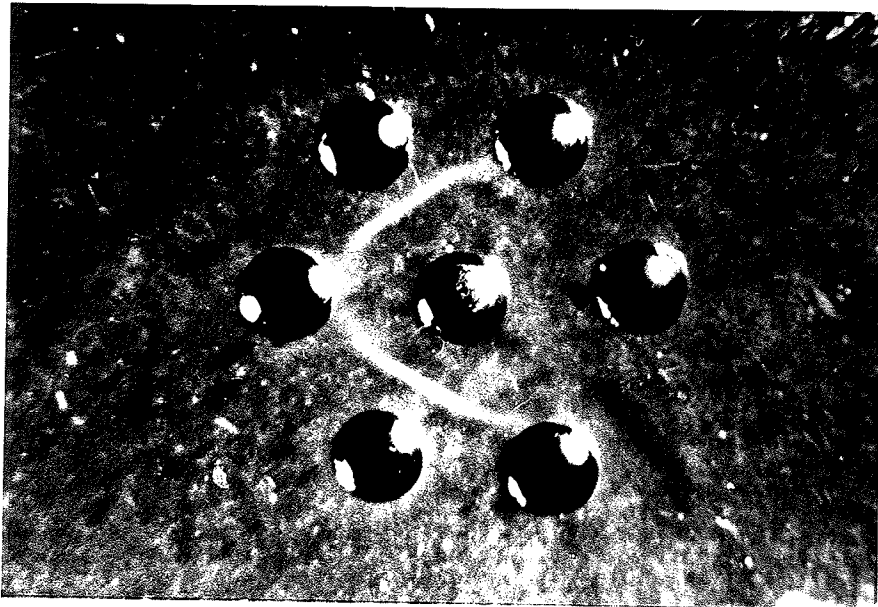
"a"

"b"

specific antisera did not give very good results as the particles seemed

to be masked by the antisera so that it was almost impossible to recognize

the particles in aggregates.   However, visible aggregates were obtained in

microtiter cups upon incubation of leukemic systems and not in control

systems.   Results correlated very well with the results of immunodiffusion

in agarose.

C.   Ferritin Studies

Aggregates of ferritin-labelled particles were seen in many of

the leukemic specimens and none in the controls.   The direct test appeared

to work better than the indirect test, probably because the two layers of

antisera make the labelling reaction less specific in the indirect test.

In the direct test, only antisera to L4S, L4L and the broad

spectrum normal antigen were conjugated.   So studies were made only with

these preparations.   Labelled particles were found in large aggregates in

the homologous systems of L4L and L4S.   The same intensity of labelling

was obtained when L4L and L4S were cross-reacted, indicating the presence

of specific antigens associated with these two human leukemic preparations.

Labelling with the normal antigen was negative in both L4S and L4L.

In the indirect test, L6L, L7S, L7L and L8L were intensely labelled

when reacted with homologous antisera or cross-reacted with each other. But

reactions with other samples were reduced in intensity.   The presence of

free ferritin particles in the conjugate preparation contributed (in a

small way) to the non-specificity of reactions which were originally quite

weak.   Since the molecular weight of these particles was very close to

that of the conjugate, it was extremely difficult to separate these materials.

Figure 7 shows the ferritin labelled aggregates that were seen

Figure 7. Immunoferritin studies of leukemic and
           non-leukemic specimens. Particulate
           antigen obtained from L-4 liver labelled
           with its homologous ferritin-conjugated
           antiserum. (27,300x) Below : non-leukemic
           antigen preparation with ferritin-labelled
           antisera to L-4 liver.
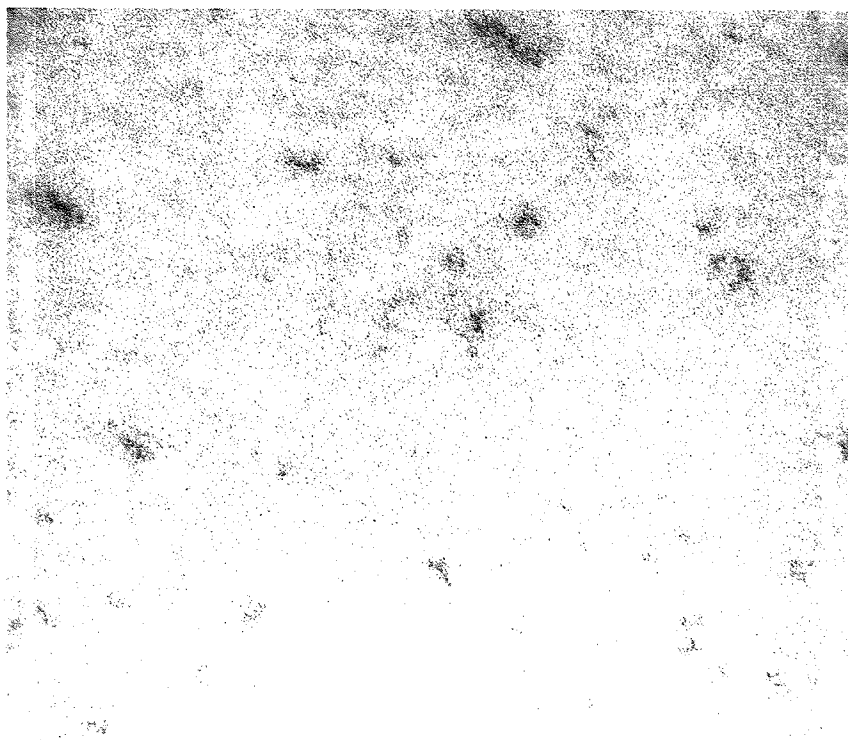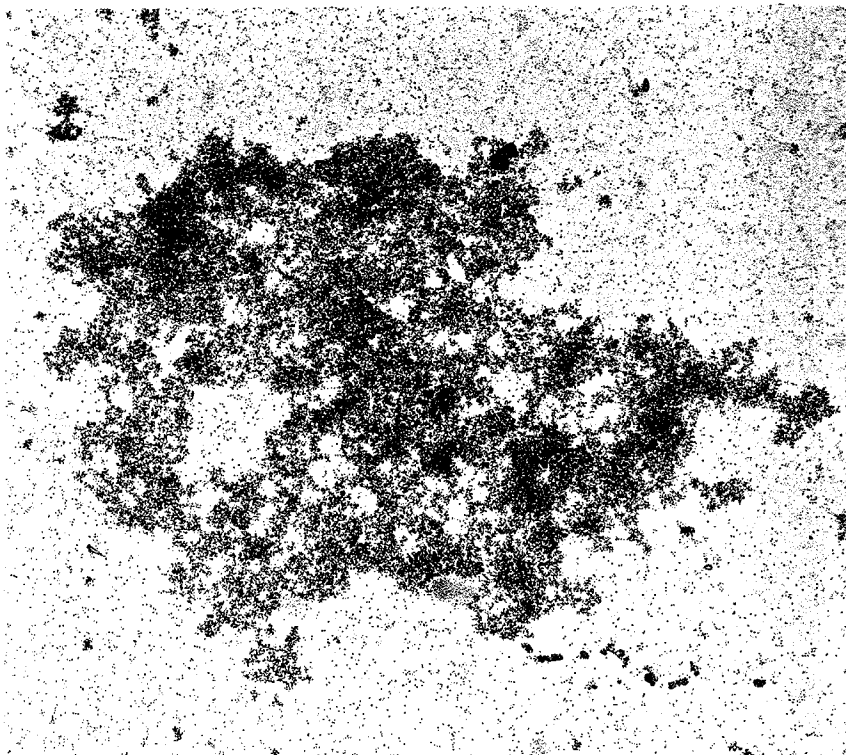
only in the leukemic preparations and not with normal preparations.

Blocking controls confirmed the specificity of these reactions.

.   On the whole, the immunoferritin studies served to confirm that

the leukemia antigen was particulate in character, whereas the immuno-

diffusion tests could have included the reactions of both particulate

and soluble antigens. It was also found that the immunoferritin test was

less sensitive, mainly as a result of greater uncertainty due to non-

specific background. But with time, the technique should prove to be a

most sensitive and useful one in immuno-electron microscopy studies.

IV.   CROSS REACTION STUDIES.

All cross-reactions of human leukemic material against animal

leukemic preparations were negative. Sonically disrupted RSV (RAV-I)

reacted with its own antisera but neither the antigen nor the antisera

reacted with any human leukemic or normal antigen preparations.

Immunodiffusion studies with GC and S63 murine leukemia viruses

were also negative. Leukemia antigens reacted with EB antiserum were also

negative, indicating that the particles had no immunological association

with EB viruses, which are known to be associated with Burkitt's Lymphoma

and infectious mononucleosis.

Chronic leukemic patients' sera were also reacted with the

different leukemic and normal antigen preparations. Again results were

negative. This may be attributed to the fact that many leukemic patients

have defective immune mechanisms as a result of the disease and were there-

fore unable to respond to the leukemic antigen if present. Another

possibility was that these patients were under immuno-suppressive drugs

and were therefore immunologically unresponsive. (Smith et al, 1968)

This was also in accordance with the findings of Kamiya (1969) who used passive cutaneous anaphylaxis and immune adherence to detect antibodies to leukemic antigens from leukemic leucocytes and brain tissue. No positive reaction was obtained with the patients' sera but a positive reaction was evident in the sera of leukemic patients' families. Benyesh-Melnick (1966) also reported similar findings.

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

As revealed by the experiments reported above, hexagonal virus-like particles with a possible immunological specificity to leukemia were recovered from livers and spleens of human leukemic patients, regardless of the clinical form of leukemia. These findings are in accordance with those of Fink et al (1968) who reported specific immunofluorescence reactions of antiserum to C-type particles produced in rabbits with the nuclear and cytoplasmic components of leukemic bone marrow and buffy coats. The consistent presence of these particles in human leukemic materials, as well as their immunologic specificity strongly indicates that they are associated in some ways with the disease.

Whether these virus-like particles play a fully etiological role in human leukemia or whether they are coincidental bodies cannot be answered easily. Unlike investigations of animal leukemias, the concentration of these particles cannot be increased in inbred populations such as in AKR high incidence leukemic mice. Most important of all, an agent which has been determined by indirect and circumstantial evidences to be associated with human leukemia, cannot be reinoculated into the original host. Thus, Koch's postulates cannot be fully satisfied.

However, experiments by Izawa et al (1966) gave an interesting insight into the role of these particles in human leukemia. Human volunteers were injected with leukemic bone marrow aspirates, in order to obtain anti-leukemia human sera, which were subsequently labelled with I^{131} and used to demonstrate the specific leukemic antigens in pathological cells of leukemic patients. However, if these particles were the sole factor responsible for human leukemia, these volunteers would have contracted the disease. Instead they remained healthy and produced circulating antibodies

to the leukemic bone marrow. This appears to be good evidence in favour

of the theory that oncogenicity is not a property of any virus per se,

but rather reflects a special type of virus-host interaction.

One aspect of this interaction that has been extensively studied

is genetic susceptibility. The concordance of leukemia in twins and

the high incidence of leukemia in children with Down's syndrome are

examples of the genetic predisposition necessary for the causal agent

to begin its infecting process. Indeed, the Philadelphia (Ph') and

Christchurch (Ch') chromosomes have been specifically associated

with human leukemia. (Cowdry,1968)

Another aspect of it is that these viruses,capable of initiating

the leukomogenic process, may be endogenous in character remaining

in a latent form in normal individuals. Some form of stimuli such as

irradiation,chemicals or subtle changes in the physiological state of

the host, may trigger the viruses into an activated form. There is

also the possibility that though the viruses are activated, the host

cell may lack the biochemical capability to support the complete

maturation of the viruses to an infectious form.

Recent evidence on the relationship between blocking and de-

blocking sera and cancer has revealed another aspect of the special

types of virus-host interaction. Hellstrom et al (1969,1971) showed

that there are certain specific serum factors in a host carrying

progressively growing tumors, that protect the tumor cells from

destruction by the host's immune lymphocytes,thus allowing the tumor

cells to proliferate. The nature of these serum factors has not been

elucidated. Subsequent experiments with the sera of patients who had

become clinically tumor-free have shown that their sera were capable

of " de-blocking " the blocking effect of sera from patients carrying

the same type of tumor. This phenomenon provides researchers with

a rational basis for the application of immunotherapy.

The work of Johansson and Klein (1970) has shown that IgM-kappa

immunoglobulins were located on the surface of cells from a patient

chronic lymphocytic leukemia. Yoshida (1970) detected by immune

adherence autoantibodies to human leukemic cell membrane,showing that

the blocking and de-blocking phenomenon may also be true in leukemia.

Indeed if infectious mononucleosis and leukemia were to have a common

etiology as is often proposed, the former may simply represent the

infectious process in an immunologically competent host,where instead

of blocking antibodies complete antibodies are formed,resulting in

a self-limiting disease. (Benyesh-Melnick,1968) Leukemia represents

the infectious precess in persons with impaired cell-mediated or

humoral immunity, where the infection is perpetuated by the host's

own dysfunction.

In this research work, particulate antigens associated with

human leukemia have been extracted and partially purified. These were

not found in the normal controls examined,although it must be said

that only a relatively small number of controls were examined due to

the limitation on the time and size of the project. It would not be

surprising if these virus-like particles were present in low concentration

in a certain percentage of the normal population and have not manifested

themselves due to reasons previously discussed.

The production of specific antisera to these particulate antigens

facilitated the immunological characterization of these particles.

Regardless of the forms of leukemia, the antigens appear to be immuno-
logically identical, thus implying the possibility of a single
etiological agent with diversified forms of manifestations. The cross-
reaction studies of human leukemic material against avian,murine and
feline leukemic antigens and antisera were all negative , indicating
that the human leukemic agent is probably not related to all the other
leukemic agents and that its different infectious process has made it
so difficult to characterize and elucidate even though so much more
is known about the other leukemias.

To establish the role of these virus-like particles in the
etiology of human leukemia, the partially purified antigen preparation
obtained can be used to sensitize human lymphocytes in vitro. Using
blocking sera from acute leukemic patients, de-blocking sera from
leukemic patients in remission and leukemic cells in culture as antigen,
the destruction of leukemic cells by these sensitized lymphocytes will
be an indication that these virus-like particles may possibly be the
causative agent of human leukemia. Once the role of these particles
are established,in vitro systems can be set up using leukemic and
non-leukemic cells in culture and the infective process of these agents
may be elucidated. Moreover, the purified particulate antigen will be
useful in studying host responses in leukemia such as the blocking and
de-blocking phenomena . Lymphocytes sensitized with the particulate
antigen can be used not only for immunotherapy but also for an in vitro
assessment of the success of the therapy. These immunological studies
may well provide important clues to the understanding of the human
leukomogenic process.

SUMMARY

SUMMARY

The aim of this research project was to isolate and immunologically characterize particulate antigens occurring in human leukemic tissues.

Particulate antigens were prepared from eight leukemic autopsy specimens of liver and spleen by the modified method of Greenspan et al (1964) and of Kwapinski (1969). Identical procedures have been used for preparing non-leukemic specimens as controls. These preparations were partially purified by continuous particle electrophoresis and isopycnic centrifugation using sodium citrate gradients. Virus-like particles (C-type) of approximately 100 nm in diameter were found in a single band with an average density of 1.18 g/c.c. (murine leukemia viruses band from 1.16 to 1.18 g/c.c., Brown et al, 1967) No particles were observed in the non-leukemic controls even after a ten-fold concentration.

Antisera to these partially purified antigen preparations were produced in rabbits. Immunoglobulins from these antisera were prepared and used in micro-precipitin tests in electron microscopy. These virus-like particles in leukemic preparations clustered only with leukemic antisera and not with non-leukemic antisera.

By using guinea-pig anti-rabbit sera immunoglobulins, labelled with ferritin, indirect immunoferritin tests were performed. Again, leukemia specific reactions were demonstrated in electron microscopy. Ferritin particles were seen with clusters of leukemic antigen and antisera but not with normal controls. The blocking or inhibition test was also performed to confirm these results.

Cross-reaction studies of the leukemic and non-leukemic preparations were performed with Rous Sarcoma Virus - Rous Associated Virus I, murine

leukemia viruses GC and S63; antisera to Moloney mouse leukemia virus, Rauscher mouse leukemia virus and Rickard feline leukemia virus. These viruses were tested in immunodiffusion first as whole viruses, then disrupted and retested. All cross-reactions in immunodiffusion were negative. Reaction of the leukemic and non-leukemic antigens with a human serum containing a high titer to Epstein-Barr virus was also negative.

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APPENDIX

PHOSPHATE-BUFFERED SALINE   (PBS)

Stock Solutions:

Solution A = 0.2M NaH_2PO_4 ($NaH_2PO_4 \cdot H_2O$, 27.6 g/1.)

Solution B = 0.2M Na_2HPO_4 ($Na_2HPO_4 \cdot 7H_2O$, 53.65 g/1.)

Saline   = 0.15 M NaCl   (NaCl, 8.5 g/1.)

0.01M PBS, pH 7.0:

16.5 ml of Solution A

33.5 ml of Solution B

8.5 gm of NaCl

Distilled water to 1 litre.

0.05M phosphate buffer, pH 7.5:

80 ml of Solution A

420 ml of Solution B

Distilled water to 1 litre.

BORATE-BUFFERED SALINE

Borate Buffer:-

Boric acid	6.184 gm
Borax (Na$_2$B$_4$O$_7$.10 H$_2$O)	9.536 gm
Sodium chloride	4.384 gm

Distilled water to 1 litre, pH 8.4-8.5

Borate saline solution:-

5 parts of borate buffer to 95 parts of saline.

TRIS-HCl BUFFER FOR GEL FILTRATION

Tris	36.3 gm
Sodium Chloride	175.5 gm
Hydrochloric acid (0.2M)	804 ml
Distilled water	2196 ml

pH = 8.0 at 25° C