MELANOMA TUMOR CELL SPECIFIC ANTIGENS ASSOCIATED WITH MICROSOMES PURIFIED FROM HUMAN MELANOMA

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ABSTRACT

MELANOMA TUMOR CELL SPECIFIC ANTIGENS ASSOCIATED WITH MICROSOMES PURIFIED FROM HUMAN MELANOMA

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The isolation and characterization of nascent melanoma-specific antigens associated with the rough endoplasmic reticulum of melanoma cells from ten malignant melanoma patients are discussed.

Partially purified IgG's obtained from highly positive melanoma patients sera were used to prepare affinity columns with Sepharose 4 B. RER membranes obtained by density gradient centrifugation of melanoma cells were further fractionated after treatment with puromycin and sodium deoxycholate to give ribosome-free, reconstituted microsomal membranes. These membranes were solubilized by treatment with Triton X-100 and used as the source of melanoma antigens. An allogeneic antigenic fraction of MW 64,000 $\pm$ 3,000 daltons has been isolated from solubilized microsomal membranes from five melanoma patients and also shown to be present on the microsomal membrane of an additional five melanoma patients. An antigen of similar molecular weight was found to be present in the immune complexes isolated from negative serum of a malignant melanoma patient.

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INTRODUCTION

INTRODUCTORY REMARKS

Cancer is a neoplastic disease which has afflicted mankind for centuries and its presence is being made known rather dramatically as the number of cancer patients is rapidly on the rise. Recent reports estimate that some 50 million people, at present alive in the United States, will develop cancer and, of these, almost 34 million will die of this disease unless more adequate treatment becomes available in the immediate future (7). Presently, there is a myriad of diverse physical, chemical and biological agents capable of transforming normal cells into neoplastic growths and as a result combined and ceaseless efforts are required by those possessing the knowledge and skills of the different disciplines, if an understanding and elimination of this urgent problem are to be achieved.

HISTORICAL OUTLOOK

According to historical annals, the first accredited mention of melanoma was by Hippocrates in the fifth century B.C. Also there is the legendary rumor that melanoma, earlier known as "black cancer" was mentioned in the Ebers Papyrus discovered by George Ebers in 1872 and dates back to about 1502 B.C. Later translation into English of this historical document by Bryan (1931) and Ebell (1937) makes no mention of a neoplasm identifiable by description as melanoma. However a number of references to "fatal black tumors with metastases and black fluid in the body" are encountered in European literature between 1650 and 1760 (1). Identification of this group of neoplasm nonetheless, has often been attributed to the French physician Laennec, who is said to have discussed "la melanose" before the Faculty of Medicine in Paris in 1806 (2). Robert Carswell, in 1838 first employed the term melanoma to designate these pigmented malignant tumors (3). By the middle of the nineteenth century considerable interest was generated and many observers became aware of the variable natural history of this pigmented tumor and speculated on possible explanations as to its origin (4,5). These observers recognized that the tumor presents itself as nodules in the lymph nodes but erroneously attributed it to de novo origin from lymphoid tissue. The most outstanding advocate at this time

was Pemberton, who in 1858 performed the first surgical operation for melanoma, removing the suspected lymph nodes (2). In 1907 Sampson - Handley implicated the role of the host in the development and natural history of melanoma accompanied by lymphatic spread and also provided evidence of regression in this type of neoplasm (6). These observations had tremendous impact on researchers at the time and it paved the way for a whole new era of research into this neoplastic disease.

DISTINGUISHING CHARACTERISTICS OF TUMOR CELLS

Tumor cells are classified as either benign or malignant, the former comprising those cells of tumor that remain localized at the site of origin in the host. Malignant cells are distinguished clinically by their ability to invade underlying tissues and to form small clumps of cells which become detached from the tumor mass and move or metastasize through the blood and lymph vessels to distant sites where new tumors may arise. Tumor cells, whether benign or malignant, are persistently altered cells which multiply true to type and against the growth of which there appears to be no adequate control in the host. This implies that during transition from normal to tumor cells some profound and heritable changes occur thus allowing the tumor cells to determine their own pattern of activities largely irrespective of the control mechanisms

which precisely govern the growth of all normal cells in an organism. This newly acquired property is known as autonomy and is the most important single characteristic of tumor cells. Another characteristic of both benign and malignant cells is that of transplantability. That is, when tumor cells are removed from a host or taken from tissue culture medium and transplanted into an appropriate recipient of the same species they develop into tumorous growths identical to that from which the tumor cells were obtained. Whether or not transplantation of tumor cells can occur depends, in part at least, on the degree of autonomy those cells have achieved. Acquisition of autonomy, incidentally, is not a fixed and unvarying process but has many gradations ranging from very slow to very rapidly growing tumor cell types. Even some of the fastest growing tumor cells do not grow as rapidly as certain normal cells. When, for example three quarters of a normal rat liver is removed surgically, the remaining liver cells will regenerate the complete organ in about two weeks time. Comparable growth rate is seldom achieved by tumors of the liver (7). Consequently it is not the rate of growth that is critical to the tumor problem but rather the development of a capacity for unrestrained or autonomous growth.

Another important characteristic of tumor cells is their lack of perfect form and function inherent to all

normal cells. This property is known as anaplasia and represents a dedifferentiation of the tumor cells (8). Thrpugh the process of dedifferentiation the tumor cells revert to embryonic type cells in so far as they achieve the capacity to produce certain proteins (fetal antigens) which are characteristics of embryonic cells only and not of the somatic cells from which the tumors were derived. Tumor cells bear little or no similarity to embryonic cells as far as their growth characteristics are concerned since, in their most undifferentiated form, normal embryonic cells respond completely to the control mechanisms provided by the organism and by individual cells whereas dedifferentiated tumor cells show a poor response to these controls.

IMMUNOLOGICAL CONSIDERATIONS IN TUMORIGENESIS

The immune system in any organism comprises a complex interaction of three classes of white blood cells whose main function has been attributed to the protection of the organism against invasion from foreign substances which may be harmful to it. The first class represents a group of lymphoid cells derived from bone marrow, which subsequently differentiates giving rise to a population of small lymphocytes called B_o - lymphocytes. These B - lymphocytes further develop on contact with an antigen into plasma cells, which when mature, actively synthesize and secrete free antibody

into the blood and other body fluids (humoral antibody). This antibody binds to the circulating antigens, neutralizing them, thus enhancing their phagocytosis.

Antibodies have long been associated with the γ-globulin fraction of serum, forming a heterogenous class of molecules, now collectively called immunoglobulin. In humans, for example, five major structural classes can be distinguished: immunoglobulin G (abbreviated IgG), IgM, IgA, IgD and IgE. Of these the IgG is the predominant class of antibody representing 80% of the total immunoglobulins with a concentration between 8 - 16 mg / ml in normal serum. It has a molecular weight around 150,000 daltons (sedimentation coefficient 7_S) and can be split by papain into three fragments, two of which are identical. These two identical univalent (one antigen binding site) fragments are capable of combining with antigens to form soluble complexes which do not precipitate out of solution and thus called F_{ab} (fragment antigen binding). The third fragment does not possess the capacity to bind antigen but crystallizes out of solution and is termed F_c (fragment crystallizable). The F_c fragment can be cleaved from the rest of the antibody by another proteolytic enzyme pepsin, this time producing one large divalent antigen binding fragment $F(ab')_2$.

IgM form the next important class of immunoglobulin. It represents 6% of the total immunoglobulin and is found at

a concentration of 0.5 - 2.0 mg / ml of normal serum. IgM is the largest of the immunoglobulins having a molecular weight of 900,000 daltons (sedimentation coefficient 19_S) and consist of five tetrapeptide subunits. The other immunoglobulins represent a group of antibodies about which not much is known. Of this group IgA seems to be the most important forming some 13% of the total immunoglobulins and found at a concentration of 1.4 - 4 mg / ml in normal serum.

Another class of cells involved in the immune response is the large mononuclear monocytes called macrophages. They play a central role in the induction of an immune response. Macrophages are capable of taking up free antigen which they partially degrade and adsorb on their surfaces where it is believed to be in a highly immunogenetic state. In this state they are poised for cooperation with the lymphocytes. Specially designated cells of both lymphoid populations then attach themselves to different determinants on these antigens bringing about close contact between the bone marrow derived cells and the thymus dependent cells. This cellular interaction occurs for a sufficient period of time permitting passage of information from the thymic to the bone marrow cells. As a result of this encounter a signal is transferred to the B - cells, probably in the form of a change in surface

membrane configuration, resulting in the activation of the synthetic mechanism of the B - cells causing them to differentiate and proliferate into plasma cells with the concomitant production of specific antibodies (9).

Macrophages are believed to be able to recognize cancer cells and other foreign substances through what is commonly known as a recognition factor. This substance has been shown to be an alpha-globulin and found in serum (10). Current belief is that this recognition factor forms complexes with the tumor cells enabling them to be easily recognized by the macrophages and subsequently destroyed through phagocytosis.

The recognition and destruction of cancer cells are not only accomplished by the macrophages but also by a special class of T - lymphocytes known as killer T - lymphocytes which are primarily involved in immunological surveillance of the cells and tissue of an organism. According to the theory of immunological surveillance these killer T - cells are capable of recognizing abnormalities in the transformed cell surface and this renders these cells vulnerable to destruction. Exactly what component on the transformed cell is recognized by the T - cells is not fully known but the current belief points towards the involvement of the histocompatibility antigen (HLA) (11). This antigen is defined as any molecule that differs from

individual to individual and is recognized in graft rejection. HLA in humans is a complex consisting of one or more light polypeptide chains and one or more heavy chains. The heavy chain is variable with respect to amino acid composition whereas the light chain is constant and well characterized. The light chain is beta 2 - microglobulin, manufactured by nearly every cell in the body and resembles the constant domain of antibodies in many of its structural features.

Two mechanisms have been postulated to explain the role of the histocompatibility antigen in the destruction of abnormal cells by killer T - lymphocytes (12).

The first hypothesis suggests that the killer lymphocytes have two kinds of receptor: one receptor binding to the HLA antigen with the second receptor binding to the abnormal (viral or tumor) antigen. For destruction of the transformed cells to occur, according to this theory, both receptors must bind to the surface antigens. The other and more favourable hypothesis suggests that the killer cell has only one type of receptor which selectively binds to a hybrid antigen consisting of an histocompatibility antigen bound to the viral or tumor specific antigen, thereby recognizing this cell as being abnormal and ultimately destroying it.

STAGING OF MELANOMA: MOLES TO DISSEMINATED MALIGNANT MELANOMA

Of the many varieties of cancer, melanoma represents one of the commonest neoplastic process known to man, having only a few rivals in the field of oncology and as a result represents an ideal group of tumors for studying the relationship between neoplastic transformation and the immunological response elicited.

Cancer is divided into two large groups: sarcomas and carcinomas. Sarcomas are defined as malignant neoplasms which arise in tissue of mesodermal origin such as connective tissue, bone, cartilage and striated muscle whereas carcinomas are tumors which originate from epithelial cells. Melanoma belongs to the latter group and designates tumors arising from melanocytes, which are cells that produce the pigment melanin and located in abundance between the dermis and epidermis of the skin, mucous membranes and choroid of the eye.

In the earliest stage of this neoplasm there is a benign abnormal collection of melanocytes known as naevus or mole (13). Early recognition of malignancy in these naevii has been a problem diagnostically. Immunological studies indicate that the serum from patients having such early forms of the lesions do not show any detectable antibodies

against melanoma specific antigens. As the disease progresses, melanocytic invasion of the dermis occurs locally, resulting in the formation of an enlarged tumor and in some cases surface ulceration. At such a relatively early stage it may be possible to obtain samples of autologous tumor cells which can be used to carry out various immunological and histological investigations necessary for proper diagnosis. This is usually followed by surgical excision of the localized tumor mass. Stage 2 of malignant melanoma starts with recurrence of abnormal cell growth in and around the site of the original tumor which had been surgically removed or a progression of the tumor locally in the form of satellite nodules. Also during this stage metastasis in the draining lymph nodes is observed. Immunity, both cellular and humoral, is most prominent and the patient's serum is shown to contain a high titre of positive antibody, detected by means of immunological techniques such as membrane fluorescence, cytoplasmic fluorescence and complement dependent cytotoxicity (14).

In some patients during this stage of the disease a large amount of tumor may appear, grow rapidly and yet remain localized. It is possible that a whole limb, for example, to become filled with melanomatous deposits and yet remain in this state for months or even years before the first sign of disseminated tumor is detected elsewhere (15). In these patients immunological studies indicate

that the presence of circulating antibody is not related to the volume or mass of the tumor present but to the degree of localization or dissemination of the tumor (16). Such studies have also shown that sera from these patients contain relatively high levels of antibody in contra-distinction to the negative sera found in patients where metastasis is predominant even though the total tumor mass is smaller in the case of metastasis.

However as the growth of the tumor continues the level of antibodies begin to fall at variable rates leading eventually to almost no detectactable tumor specific antibody. This drop in circulating antibody, in some cases, precedes the spread of the tumor to distant parts of the body by weeks or months. This has led to widespread speculation that tumor cells may be in circulation during the entire natural history of the disease but the actual 'settling out' of the tumor cells, followed by clinical metastasis, is related to the presence or absence of circulating antibody. Some evidence for this belief comes from studies with animals indicating the presence of immunologically specific factors in blood and lymph which seem to prevent lung metastasis (17). In addition humoral antibodies have also been shown to exert a significant effect in controlling feline sarcoma (18).

The onset of stage 3 is marked by widespread

dissemination to distant organs and tissue in the body, often resulting in so much metastasis that the melanin liberated into the bloodstream causes such intense pigmentation that the entire body takes on a dusky coloured hue. Large quantities of melanin may also be found in the urine, which in many cases turns black. Studies over the years have shown that there is almost no tissue or organ of the body which has not been afflicted with metastatic malignant melanoma (19). Organs, such as the gall bladder, small intestine, the mucosa of the stomach and the spleen which are not commonly involved in neoplastic diseases of other types very often are found to be affected by metastatic melanoma. At this advanced stage of the disease tumor associated immunity is decreased to the point of total unrecognition. Reports have indicated that cellular immunity may be present but constantly being suppressed (20) whereas humoral antibody production at this stage is no longer detectable (21) except for antibodies directed against nucleolar components as reported by McBride and co-workers (22).

In spite of this dramatic decline in host immunological resistance there have been many well documented cases of complete spontaneous regression and subsequent recovery of melanoma patients, some of whom were in the very advanced stages of the disease. In fact it has been shown that the

spontaneous regression rate in melanoma accounts for 15% of all cases of spontaneous regression of human tumors whereas this type of cancer represents only 1 - 3% of all malignancy (23). Regression of this type of cancer has also been achieved to variable extents through clinical treatment of patients with immunotherapy and chemotherapy accompanied by surgery to remove extra tumor load. Another common form of treatment presently used is radiotherapy.

At present these different forms of therapy are the only clinical weapons available to combat this and other types of neoplastic diseases and as a result the need is always there for much more intensive study to develop new and more effective means to eliminate this dreadful disease.

TUMOR SPECIFICITY BETWEEN ANTIBODY - ANTIGEN REACTION IN MELANOMA:

One area of tumor immunology that is currently receiving considerable attention is the understanding of the nature and distribution of tumor antigens. Such an understanding is necessary since it will obviously shed light on reasons for the failure of the immune system during the advanced stage of melanoma.

The distribution of melanoma specific and melanoma associated antigens have recently been uncovered due mainly

to the work of Lewis et al (24). They showed by means of immunofluorescence and complement dependent cytotoxicity that in melanoma there are at least six groups of antigen namely (i) autologous membrane, (ii) autologous cytoplasm, (iii) allogeneic membrane, (iv) allogeneic cytoplasm, (v) autologous nucleolar (22) and (vi) fetal or carcinoembryonic (25). Liberation of one or more of these antigens into the blood stream by the growing tumor can often result in the formation of immune complexes with the circulating antibodies and may be responsible for the decrease in the level of tumor specific antibodies in circulation as the disease progresses. The presence of circulating antigens have been demonstrated clearly and these antigens were shown to be able to transform significantly the patients' lymphocytes (26). The liberation of cytoplasmic antigen is very likely related to the volume or mass of the tumor or degree of necrosis whereas the shedding of membrane surface antigens could well be mainly responsible for the neutralization of circulating tumor specific antibodies (24). However not much is known as yet about these occurences but with future research along these lines it should be possible to demonstrate and categorize the different antigens and more significantly to relate them in more detail to the patient staging of this disease.

In order to demonstrate differences and similarities,

if any, in these groups of tumor specific antigens, it is crucial that evidence be provided showing conclusively that these are in fact tumor specific and not tumor associated antigens. One of the earliest methods used in determining the nature of tumor antigens consisted of a form of complement dependent cytotoxicity. With this test antibodies specific for a particular tumor, in this case, melanoma were detected. This method involved the use of teflon rings on which a glass coverslip was attached as designated by Pulvertaft (27). Freshly prepared cells were allowed to attach to the underside of the coverslip followed by inversion of the apparatus so that dead cells or debris are washed away with the tissue culture medium. When patient serum and serum complement were added to these coverslips the tumor cells were no longer able to remain attached to the coverslips and were easily washed off, thus showing the effect of specific antibodies in the serum. Control test with other types of tumors and normal fibroblasts grown in tissue culture showed no such effects (28).

Another common and reliable method used to determine melanoma antigen - antibody specificity is immunofluorescence. In melanoma, two components of the cell have been used to detect such reactions. The first involves liquid nitrogen snap - frozen preparations of viable melanoma cells. This procedure exposes the cytoplasmic contents

of the cell, which when treated with serum and fluorescent conjugated antibodies, results in the formation of antigen - antibody complexes which fluoresce when viewed under the light microscope equipped with special light filters. By this technique it has been shown that the cytoplasmic contents appear to contain group specific antigens which can bind to positive serum from any melanoma patient (28). However, additional work has shown that there are more than one group of antigens in the cytoplasm (24) and it is likely that they may elicit antibody production at different stages in the course of the disease.

The other component of melanoma cells utilized to demonstrate antigen - antibody specificity is the plasma membrane. This technique is basically similar to the cytoplasmic immunofluorescence but instead a viable single cell suspension is used (29). In this system a high titre of melanoma specific antibodies was observed in the autologous situation but only a low titre in the allogeneic (cross - reacting) serum. Often when carrying out these immunofluorescent tests, serum absorptions are performed and in this way it is possible to check the specificity of a particular antigen. In melanoma for instance, when serum was absorbed out with a variety of homogenates including melanin granules, normal pigmented skin and

non-pigmented skin, Lewis and Phillips (29) showed that the titre of antibody determined by immunofluorescence remained the same as before absorption, indicating again the presence of melanoma specific antigens located both on the surface of the cell and in the cytoplasm.

Are these tumor - specific antigens in any way responsible for the lack of antibody detection during the disseminated stage of malignant melanoma? Or are these melanoma patients immunologically abnormal and as a result do not respond adequately to the growing tumor? Initially these questions were asked and after close examination of different groups of melanoma patients and normal individuals it was shown that the only detectable difference between normal and malignant patients was the absence of tumor specific antibody in the latter group (30). There was no indication of any type of immunological abnormality in the melanoma patients. Auto - immunizations of malignant melanoma patients with irradiated autologous tumor cells have shown that in the majority of cases there was a rapid rise in detectable circulating antibody which lasted for up to several weeks in some cases, followed by a dramatic decline to no detectable antibody characteristic of a secondary type of immune response (31). The same kind of results were obtained for cellular response in the form of cytotoxic lymphocytes using essentially the same technique

and providing further evidence to support the general belief that melanoma patients are not immunologically abnormal.

On the other hand when high titre positive antibody serum was mixed with equal volumes of serum either from normal patients or from other melanoma patients as well as phosphate buffered saline and these mixtures subjected to both membrane and cytoplasmic immunofluorescence no appreciable difference in the intensity of immunofluorescence was observed (32). But when positive post - immunized serum was mixed with autologous negative pre-immunized serum no immunofluorescence was observed. This effect was interpreted to be due to complete blocking of antibodies by some substance associated with the immunoglobulin fraction of serum.

Isolation of this immunoglobulin fraction from both negative and positive serum by ammonium dextran sulphate followed by separation of the immunoglobulin classes and testing each fraction using immunodiffusion on cellulose acetate membrane showed immunoprecipitation reaction between the IgG fraction of the positive and negative serum. Also formalized sheep erythrocytes coated with the gamma globulin of the positive serum were agglutinated only by IgG fraction of the negative serum and not by the numerous controls used. The conclusion arrived at from

these studies was that the blocking substance was either antibody - antigen complex with antigen excess or an idiotypic IgG antibody.

Evidence supporting any one of these claims is by no means in abundance but recently Hartmann and Lewis (33) were able to detect anti - IgG in the serum of melanoma patients by a passive haemagglutination test. The anti - IgG containing serum reacted also with autologous and allogeneic F_{ab} and $F(ab')_2$ human IgG fragments. This study and others also showed that the anti - IgG found in the serum of melanoma patients at certain stages of the disease do have a pronounced effect on the anti - cytoplasmic antibody but not on the autologous anti - membrane antibody (34). This strongly suggests the presence of two types of anti - antibody; an anti - idiotypic type directed against autologous anti - membrane tumor antibody and an anti - allotypic directed against allogeneic anti - cytoplasm tumor antibody (35). How these various antibodies and anti - antibodies relate to the failure of the immune system has yet to be determined.

There are several lines of approach open in the study of the exact nature of this blocking substance whether it be anti - antibody or antibody - antigen complex. Bioabsorption or affinity chromatography in which the bioabsorbent material is coupled to melanoma specific antibody is one such

approach. Antigen can then be passed through the column and isolated in purified form.

Previous studies that have been carried out on surface membrane antigens in human malignant melanoma (14, 29, 36) were designed to obtain information regarding the formation of antigen - antibody complexes and the decrease in titre of circulating antitumor antibody, possibly due to the formation of these complexes. Presently, more intensive studies on these surface membrane antigens are being carried out by different groups involved in tumor immunology. On the other hand the cytoplasmic antigens have received relatively little attention, even though this group of antigens has been shown to be widely cross - reactive (28). However recent investigation by Preddie and Van Allestyne (personal Communication) using microsomes (RER) isolated from melanoma cells revealed a high level of autologous cytoplasmic antigenic activity associated with this subcellular fraction. In light of this a thorough understanding of the relationship between the membrane and cytoplasmic antigens is warranted since most membrane proteins (including antigens) originate from the cytoplasm.

The origin of membrane bound secretory proteins has been shown conclusively to be on the rough endoplasmic reticulum (37, 38). Nasoent proteins are manufactured on the ribosomes which are bound to the membranes of the endoplasmic

reticulum. These newly synthesized proteins are then vectorially discharged into the cisternal space or inner cavity of the reticulum (43). From here they move to the Golgi apparatus where they are concentrated and then packaged into membrane bound vesicles (post - Golgi vesicles) which are subsequently transported to the plasma membrane where they fuse, discharge their contents and eventually become part of the cell membrane. En route to the plasma membrane these proteins are modified and in most cases only the final products are discharged. Very often the products contained within these membrane bound vesicles are also incorporated into the cell membrane forming an integral part of it and creating a continuum between nascent polypeptide synthesized on the rough endoplasmic reticulum and finished products incorporated in the plasma membranes.

Based on these observations, the approach taken in this project towards an understanding of the immune mechanism in this type of tumor was to isolate and characterize antigenic molecules localized in the RER of human malignant melanoma cells. The experiments carried out were designed with the following objectives: (a) to localize the sub-cellular cytoplasmic component with the highest melanoma specific antigenic activity and isolate this component in purified form and use it as a source of antigen

(b) to isolate antigen(s) from this subcellular component using affinity chromatography columns prepared from Sepharose 4B to which partially purified IgG obtained from melanoma patient serum are covalently coupled (c) to characterize the antigen(s) isolated immunologically and chemically and (d) to determine whether there exists any immunological and/or chemical similarity between antigens isolated from the sub-cellular component and antigen(s) isolated from immune complexes of negative serum of malignant melanoma patients.

MATERIALS AND METHODS

1. MALIGNANT MELANOAMA TUMOR TISSUE

Human malignant melanoma tissue was obtained either at surgery or at autopsy from patients at the Royal Victoria Hospital, Montreal, Quebec, and used immediately or stored at - 40° C until processed.

11. PREPARATION OF CELL SUSPENSION

Fresh tumor tissue for cell suspension was minced finely in Medium 199 (Burroughs Wellcome, Langley Park, Beckingham Kent U.K.) and filtered through four layers of sterile gauze. 2 drops of cell suspension were mixed with 2 drops of Trypan blue and transferred to haemocytometer slide. These cells were then counted using a light microscope. Only tumor cells were counted omitting red blood cells, lymphocytes etc. The stained cells were non - viable whereas the unstained cells were viable. The total tumor cell count was obtained by either counting the number of cells in one corner consisting of sixteen squares or counting more than one corner and taking the average.

Total cell count = total cells counted in one corner X dilution factor (ie 2) X 10^4 X total volume of cell suspension.

$$\text{Viability (\%)} = \frac{\text{no. of viable cells in corner}}{\text{total no. of cells in corner}} \times 100\%$$

The cell count was then adjusted to between 1 - 2 million cells / ml which was then used for the preparation of smears for immunofluorescence. Excess tumor cells were processed and stored at - 180° C until further use. For storage each ml of cell suspension was centrifuged at low speed and the supernatant decanted. To the pelleted cells were added 0.15 ml DMSO, 0.15 ml of FCS (fetal calf serum) and 0.70 ml of a mixture of Medium 199 and 10% FCS.

III. MELANOMA PATIENT SERUM

Serum samples from melanoma patients whose tumors had been processed as above and from patients with other malignant disease (teratoma and liposarcoma) as well as normal patient sera were collected in sterile containers and after dispensing into 1ml aliquots, stored at - 136° C in vapour phase liquid nitrogen.

IV. ABSORPTION OF SERUM USING NORMAL HUMAN SPLEEN CELLS

Fresh or frozen spleen cells were thawed rapidly in a 37° C water bath then transferred to corex tubes and centrifuged at low speed (500 X g). The supernatant was discarded and the pellet resuspended in sterile isotonic saline and homogenized with a teflon homogenizer. The emulsion was quickly frozen and thawed to burst any remaining cellular material. An equal volume of isotonic saline was then added to the mixture and it was spun for 1 hour at 5,000 X g and 4° C. The supernatant was discarded and the pellet washed three times with isotonic saline followed by one washing with PBS (0.01M potassium phosphate, 0.15M NaCl) pH 7.4. The resulting pellet was mixed with an equal volume of PBS and 1 ml aliquots transferred to centrifuge tubes and centrifuged for 30 minutes at 10,000 X g and 4° C. The pellets were stored at - 20° C or below (39) until used. About 1 ml of each serum to be absorbed was mixed with the above pellet in a tube and incubated for 1 hour at 37° C. During incubation the tubes were kept agitated enough to provide constant mixture between serum and absorbant without causing foaming or denaturation of the protein. After incubation the tubes were centrifuged for 30 minutes at 10,000 X g and 4° C to recover the serum. Each serum was again mixed with a fresh sample of spleen preparation and further incubated at 37° C for 30 minutes followed by overnight incubation at 4° C. Final recovery

of the serum was achieved by centrifugation for 30 minutes at 10,000 X g and 4° C.

V. CYTOPLASMIC IMMUNOFLUORESCENCE

(a) PREPARATION OF SLIDE FOR IMMUNOFLUORESCENCE: The smears (3 smears/slide), each containing approximately 30,000 cells (1 drop of 1 x 10^6 cells / ml per smear), were prepared on short haematological slides that had been previously cleaned and dried. The smears were allowed to dry at room temperature for 30 - 60 minutes. Meanwhile, a frozen mixture for use in snapfreezing the cells was prepared by slowly adding liquid nitrogen to precooled isopentane in a plastic container until a toffee - like bubbly solid was formed. This served as a temperature indicator and when the isopentane reliquified the temperature was - 160° C. At this point the air dried smears were plunged into the liquid and allowed to remain submerged for 1 minute. The slides were then removed and excess isopentane drained off leaving an icesheet over the smears. These were ready for use and were either used immediately or stored frozen at - 180°°C. Snapfreezing of cells in this way appeared to open up the cell membrane and facilitated free access to the cytoplasm by immunoglobulins. It also enhanced adhesion and flattening of the cells.

VI. DETERMINATION OF ANTIBODY TITRE IN SERUM

For this process the slides prepared above were removed from storage and thawed out at room temperature for 30 minutes. These slides were numbered and an oval spot was traced around the smear with a diamond pencil to indicate the exact location of each smear on the slide. This was followed by washing the slides with PBS pH 7.3 for 30 minutes with gentle shaking. Serum to be tested were diluted initially 1 : 4 with PBS and kept at room temperature (40). These were then applied to the smears by means of a Pasteur pipette. The application of serum was carried out only after the slides had been air dried. When serum application was completed, the slides were covered with large glass lids and incubated at room temperature for 30 minutes. Controls such as highly negative sera or PBS were treated similarly. The slides were then washed twice with PBS as before with each washing 30 minutes in duration.

Conjugated antiserum, Isomer 1 fluorescein isothiocyanate (FITC, Behringwereke. AG. Behring Institute, Germany, Lot 675 D, 3rd February '77) was prepared by dissolving the content of 1 ampoule (1 ml size) in 1 ml of doubly distilled water and 7 ml of PBS pH 7.3 (29). This was carefully applied to each smear (1 drop $\simeq$ 0.010 ml) and incubated at room temperature for an additional 30 minutes

after which time any excess conjugated antiserum remaining on the smears were gently washed off with PBS. Three twenty (3 X 20) minutes washing with PBS as before were carried out, after which, the slides were removed from the washing medium, excess buffer carefully wiped off and air dried for a few minutes. Each smear was then covered with a No.1 grade coverslip onto which one small drop of 1 : 1 Analar grade glycerol: PBS had been placed. Care was taken so that no air bubbles were trapped beneath the coverslip as this distorted fluorescent observation under the microscope.

Microscopical examination was carried out using a Wild M 20 microscope equipped with an incident light system as described by Ploem (41). This system incorporates both an exciter filter with an identical reflecting filter (dichroic). These filters were specific wave-band interference filters designed so as to excite the fluorochrome at the peak of its absorbance. It enables FITC fluorescence to be observed at 520 - 530 nm, which is the optimum range for FITC (73). Within this range the emitted fluorescence is brilliant apple green whilst the background is dull red (40).

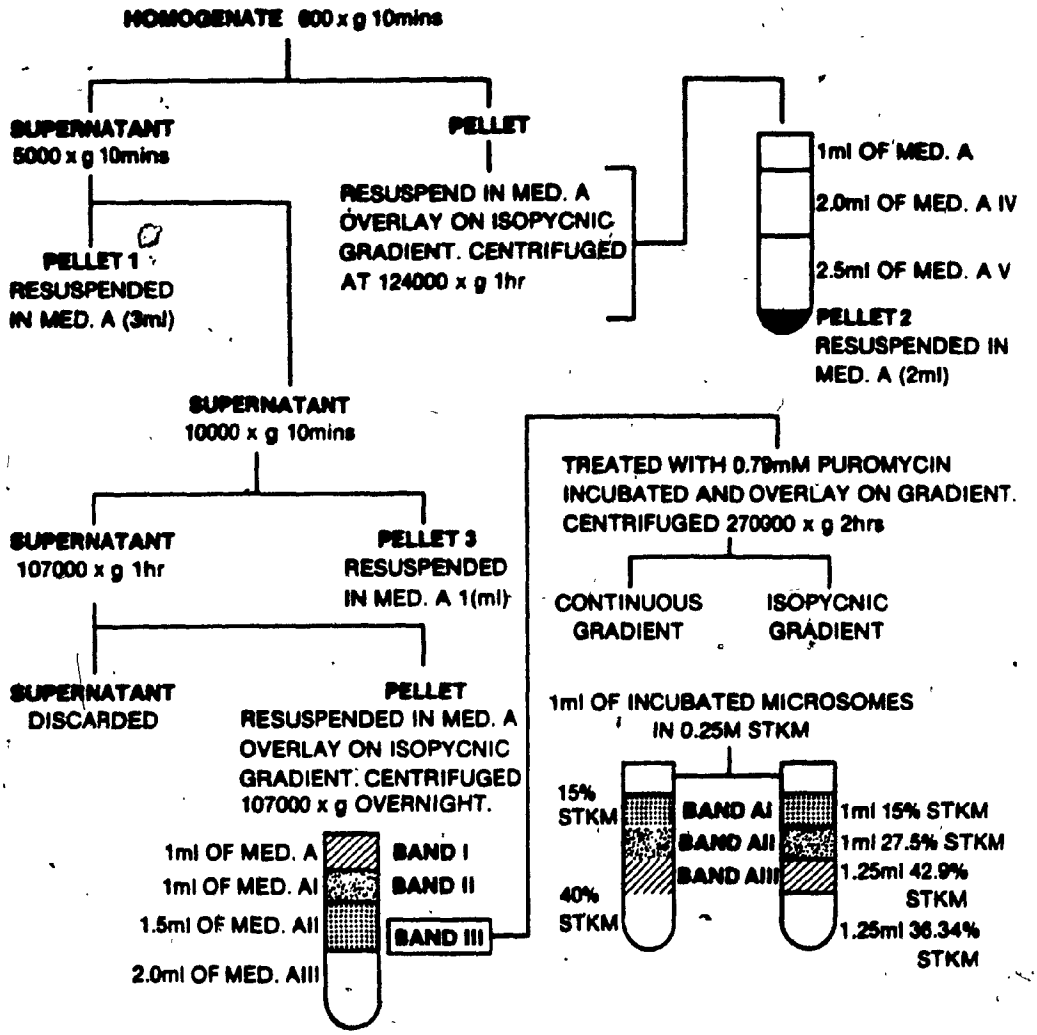
VII. PREPARATION OF SUBCELLULAR FRACTIONS

(a) FRACTIONATION OF CELLS USING DIFFERENTIAL CENTRIFUGATION:

Frozen tumor tissue was thawed rapidly in a 37° C water bath. Excess blood was filtered off with sterile gauze. The rest of the fractionation procedure were carried out in a cold room at 4° C. The tissue was then weighed, minced and ground in medium A (Med. A 0.25 M sucrose, 5 mM $MgCl_2$, 2.5 mM KCl, 5 mM β - mercaptoethanol and 100 mM Tris - HCl pH 7.5) using a mortar and pestle followed by homogenization with a loose fitting teflon homogenizer (0.004 - 0.006 inch clearance) with 10 to 12 strokes. The suspension was filtered with four layers of gauze and centrifuged as shown in Flowchart 1. Whenever needed, nuclei were prepared according to the method of Blobel and Potter (42). RER (rough endoplasmic reticulum) was prepared according to the method of Burrow and Sauer (43) with minor modification to give a purer preparation. RER was collected at the interface of the 1.35 / 2.0 M sucrose as described by Redman (44). Pellet 1,2, and 3, suspended in Medium A, Band I and Band II were each diluted to about 16 ml. with Medium A and centrifuged at 107,000 X g for 1 hour. The pellets suspended in suitable volumes of Medium A were then dialyzed against 3 or 4 changes of 0.15 M NaCl and stored frozen until further use. Band III which contained

the RER (characterized by enzyme assays, electron microscopy and chemical methods) was further subfractionated essentially according to the method of Adelman et al (45). Briefly, microsomes (RER) obtained in Flowchart 1 was diluted 1 : 4 with Medium A and centrifuged for 1 hour at 107,000 X g and 4° C using an SW 65 rotor and a Beckman Spinco Model L centrifuge. The pellet was resuspended in 0.25 M sucrose in TKM (50 mM Tris - HCl, 750 mM KCl and 5mM $MgCl_2$) buffer pH 7.5 containing 0.79 mM puromycin at a concentration of 1 - 2 mg RNA / ml as determined by the Orcinol method (45). This sucrose solution was brought to a final concentration of 1% Ribonuclease A (Sigma) and incubated for 45 to 60 minutes at room temperature. This was then layered on a 15 - 40% continuous sucrose gradient with the same concentration of TKM (50 mM Tris-HCl, 750 mM KCl and 5 mM $MgCl_2$) as the overlaying solution. Comparative experiments were carried out by overlaying the incubated microsomes on isopycnic gradients made up with 15, 27.5, 36.34 and 42.9% STKM (sucrose, 50 mM Tris - HCl, 750 mM KCl and 5 mM $MgCl_2$) prepared in 5 ml polyallomer (Beckman) tubes. These were usually centrifuged for 2 hours at ~ 270,000 X g (50,000 rpm) and 4° C in a Beckman L 50 centrifuge using an SW 65 rotor. In both cases three distinct bands and a very small pellet were visible. For the continuous gradient these bands were removed by punching a hole with a needle at the side of the tube just above

the pellet and collecting 20 - 25 drops fractions: case of the step gradient each band was removed with the aid of a Pasteur pipette. Each fraction was very carefully labelled and diluted 1 : 5 with cold distilled water and centrifuged for 2 hours at 100,000 X g and 4° C. The pellets obtained were resuspended in TKM buffer, dialyzed against 0.15 M NaCl and stored frozen at a protein concentration of about 10 mg / ml as determined by the method of Lowry et al (47). RNA concentration on each fraction was also determined. All fractions were tested for immunological activity with double diffusion Ouchterlony using both non - solubilized and 1% Triton X 100 solubilized fractions. Immunological activity was also determined using counter-current immunoelectrophoresis. The band between the 36.34 and 42.9% sucrose, Band A III, in the isopycnic gradient and the first band from the continuous gradient were always immunologically active and were shown to be the stripped rough microsomal membrane (45). In some cases the rough microsomal membranes were further treated with 0.049% sodium deoxycholate (DOC) to give a reconstituted membrane, Fraction I (FI) and cisternal content, Fraction II (F II) (48, 62). The procedure was as follows; stripped rough microsomal membrane at a concentration of about 10 mg / ml was first centrifuged for one hour at 107,000 X g and 4° C. The pellet was resuspended in 4.5 ml of buffer (2 mM $MgCl_2$, 500 mM KCl and 1 mM Tris - HCl pH 7.8) and brought to a



FLOWCHART 1. Fractionation of cells. Medium A, A I, A II, A III, A IV and A V consist 0.25, 0.86, 1.35, 2.0, 1.83 and 2.0M Sucrose respectively, each containing 5mM $MgCl_2$, 2.5mM KCl, 5mM β-mercaptoethanol and 100mM Tris - HCl pH 7.5. Pellet 1 contains Mitochondrion, Pellet 2 contains purified Nuclei and Pellet 3 contains Lysosomal Vesicles Band I contains a mixture of plasma membrane and post Golgi Vesicles, Band II contains SER and Band III contains RER. 0.25M, 15, 27.5, 36.34, 40.0 and 42.9% STKM each contains Sucrose as indicated plus 50mM Tris - HCl pH 7.5, 750mM KCl and 5mM $MgCl_2$. Band A I and A II contain Ribosomes, Band A III contains stripped RER.

final concentration of 0.049% DOC. This was incubated for 5 minutes at 37° C then diluted 1 : 4 with cold distilled water and centrifuged for 16 hours at 107,000 X g and 4° C. The pellet contained the reconstituted microsomal membrane with the cisternal space content located in supernatant. This was concentrated against Ficoll (Sigma Type 70) and both F I and F II dialyzed against 0.15 M NaCl and stored until used.

VIII. ENZYMATIC CHARACTERIZATION OF CELLULAR FRACTIONS

The main objective of the cellular fractionation performed earlier was to obtain a purer preparation of microsomes (rough endoplasmic reticulum) which was then further fractionated. In order to determine the purity of the microsomal fraction enzyme assays were carried out. Adenosine triphosphatase, (ATP ase), which is a marker enzyme for plasma membrane (49, 50), glucose 6 - phosphatase an enzyme marker for smooth endoplasmic reticulum (51, 52) and NADH cytochrome C - Reductase located mainly in the rough endoplasmic reticulum (51, 53) were assayed. These assays were carried out on four major sub - cellular fractions shown in Flowchart 1 as (a) the homogenate (b) Band I (c) Band II and (d) Band III.

(a) ADENOSINE TRIPHOSPHATASE: This assay was done according to the method of Ernster et al (7) as modified by

Post and Sen (54). The following solutions were prepared for the assay:

SOLUTION 1 — 0.05 M Na_2 EGTA (Sigma)
4.00 mM $MgCl_2$
5.00 mM NaN_3
0.05 M Tris - HCl pH 7.5
4 times (4 X) concentrated

SOLUTION 2 — 0.10 M NaCl
0.02 M KCl
4 X concentrated

SOLUTION 3 — 5.0 mM Na_2ATP (Sigma)
8 X concentrated

SOLUTION 4 — 1.0 mM OUBAIN
4 X concentrated

Into small washed tubes were added 0.15 ml of distilled water. An amount, same as the volume of the enzyme fraction to be added, was removed and 0.10 ml of solution 1 and 0.10 ml of solution 2 were added followed by the addition of (50 ug) the necessary quantity of the fraction containing the enzyme. The mixtures were gently shaken and incubated in a 37° C water bath for 2 - 3 minutes then 0.05 ml of solution 3 was added to each tube and again incubation followed at 37° C for 1 hour. Duplicate tubes were

prepared in the same way except that here solution 4 replaced solution 2 in the same quantity. After incubation the reaction was stopped by the addition of 0.1 ml of 25% TCA (trichloroacetic acid) and the amount of phosphate released was determined.

(b) PHOSPHATE DETERMINATION: To determine the amount of phosphate released in the reaction of a simple colorimetric test was carried out. This test involves the formation of a coloured complex of reduced polymolybdophosphoric acid (55). The molybdophosphoric acid is a large inorganic complex with the empirical formula m H_2O. P_2O_5. y Mo O_3 (where y = 12 or 24 in most cases). This complex is formed in an acidic solution of ammonium molybdate and phosphate ions. In the presence of ascorbic acid, some of the molybdate ions in the complex become reduced and absorb light at wavelengths of 650 -959 nm resulting in a deep blue colour. Fresh solutions of 0.5% Ammonium molybdate (Sigma) in 2 N H_2SO_4 and 2% Ascorbic Acid in water were prepared for each assay.

For the phosphate determination 0.2 ml of the ATPase phosphate solution was mixed with 2 ml of the molybdate solution and 2 ml of the ascorbic acid solution. The mixture was incubated for 20 minutes at 45° C then cooled on ice to stop the reaction. The tubes were brought to room temperature and the absorbance read at 820 nm.

(c) GLUCOSE 6 - PHOSPHATASE: This assay was done according to the method of Swanson (56, 57). For the assay the following stock solution were prepared:

SOLUTION 1 — 260 mg. of glucose 6 - phosphate, barium salt, hydrate, G - 6 P. $7H_2O$ (Sigma) suspended in 2 ml of distilled water. A minimum amount of 1 N HCl was added to completely dissolve glucose 6 - phosphate. 72 mg. of anhydrous Na_2SO_4 (0.5 mM) added and the precipitated $BaSO_4$ removed by centrifugation in an Eppendorf centrifuge. The supernatant tested for complete precipitation of $BaSO_4$ with Na_2SO_4. The supernatant brought to pH 6.5 with 50% NaOH and made to 5 ml with water.

SOLUTION 2 — 116 mg. of maleic acid (Sigma) dissolved in water and brought to pH 6.5 with NaOH and made to 10 ml.

Into small acid washed tubes 0.10 ml of water was added. From this an amount equivalent to the volume of the enzyme fraction to be added was removed and 0.2 ml solution 2 (buffer) added as well as 0.1 ml of substrate (solution 1). The tubes were shaken gently and incubated in a 37° C

water bath for 2 - 3 minutes after which 50 ug of the fractions containing the enzyme were added and further incubated at 37° C. for 15 minutes. The reaction was stopped by the addition of 0.1 ml of 25% TCA and 0.2 ml of this mixture was used for phosphate analysis as before.

(d) NADH-CYTOCHROME C REDUCTASE: This assay was performed according to the method of Hodges and Leonard (58), except the final volume in the cuvette was 1.5 ml. For the assay the following solutions were prepared:

SOLUTION 1	50 mM sodium phosphate buffer pH 7.5.
SOLUTION 2	50 mM potassium cyanide (KCN) in distilled water.
SOLUTION 3	0.45 mM cytochrome C (Sigma type VI) in 50 mM sodium phosphate pH 7.5 (solution 1).
SOLUTION 4	3.0 mM NADH (Sigma β - NADH) in solution 1.

This assay was carried out at room temperature by measuring the reduction of cytochrome C in a Beckman - DB spectrophotometer to which a recorder was attached. Whenever a recorder was not available, the change in absorbance was recorded at 10 seconds intervals. The reduction of cytochrome C was measured at 550 nm.

Into small cleaned tubes 1.3 ml of buffer (solution 1) was added and from it was removed an amount equivalent to the volume of the fraction containing the enzymes to be added. To each tube was subsequently added 0.05 ml of solution 2, 0.10 ml of solution 3 and then the enzyme fraction (50 ug of protein). The solutions in each tube gently mixed and transferred to a 1.5 ml quartz cuvette and the reaction started by the addition of 0.05 ml of the NADH solution. A blank was prepared in a similar way except no NADH was added to the cuvette, instead water was used. The rate of cytochrome C reduction was determined from the initial linear rates and the amount of cytochrome C utilized was calculated using an extinction coefficient for cytochrome C of 18.5 mM^{-1} cm^{-1} 550 nm.

IX. PREPARATION OF CHROMATOGRAPHY COLUMNS

(a) ISOLATION AND PURIFICATION OF IgG FROM MELANOMA PATIENT SERUM:

Absorbed positive melanoma patient serum were brought to 40% ammonium sulfate by the addition of cold saturated (100%) ammonium sulfate (59). The required amount of ammonium sulfate was calculated according to the formula.

$$Y = \frac{V_1 \quad S_2 - S_1}{1 - S_2}$$

where

Y = ml. of saturated $(NH_4)_2 SO_4$ to be added to V_1

V_1 = initial volume of solution at S_1

S_2 = saturation desired.

The serum containing the ammonium sulfate was gently mixed in a vortex mixer and allowed to stand for 30 minutes at 4° C. The solution was then spun in an Eppendorf 3200 clinical centrifuge for 10 minutes at maximum speed. The supernatant was discarded and the pellet resuspended in 0.01 M potassium phosphate buffer pH 7.5. This was then dialyzed for 16 hours against the same buffer. This precipitated proteins contained mainly immunoglobulins and further purification to give predominantly IgG was required.

This was achieved through anion exchange chromatogrpahy using DEAE (diethylaminoethyl) cellulose (Sigma, medium mesh 0.9 meq / g). This anion exchanger is a chemically modified cellulose, of which about 10% of the glucose moiety are alkylated with DEAE groups which when protonated carry a positive charge and have a pKa of 8.

For this process 4 gm. of DEAE cellulose was added to a 1 litre beaker filled with 0.2 M NaOH. The suspension was stirred with a glass rod and the heaviest particles were allowed to settle to the bottom of the beaker. The yellowish

opaque fines were decanted and the cellulose washed again, once with distilled water and twice with 0.2 M HCl. This was followed by repeated washings (usually 5 times) with water until the pH increased to about 5. The pH was then adjusted to 7.0 with K_2HPO_4 using a pH meter and the cellulose washed two times again with 0.01 M potassium phosphate pH 7.5. All the above washings were carried out at room temperature. After the washings were completed the cellulose was either used immediately or stored at 4° C in the same buffer.

When ready for use, the buffer was poured off until the volume of the buffer was same as the DEAE cellulose. This was stirred and packed into 1.5 cm X 30 cm columns (Pharmacia Chemicals) to a height of 20 cm and washed thoroughly with 0.01 M potassium phosphate buffer pH 7.5 until there was no change in the pH of the effluent. 40% ammonium sulfate precipitated proteins (9 - 12 mg) were carefully loaded unto the column and allowed to enter the column at a flow rate of 10 drops per minute. The top of the column was washed twice with a similar volume as that of the IgG solution added. The column was then eluted with a continuous salt gradient made from 0.005 M NaCl and 0.5 M NaCl in the same phosphate buffer (0.01 M potassium phosphate pH 7.5). 3 ml fractions were collected with an LKB 17000 Minirac automatic (Fisher Scientific) fraction collector.

Each fraction was subsequently checked for protein at 280 nm (absorption by tyrosine and tryptophan residues) and the immunologically active fraction against horse anti - human IgG (Behringwerke AG Germany) was determined by double diffusion Ouchterlony and microprecipitin. These fractions were pooled and concentrated by ultrafiltration in an Amicon chamber fitted with an XM 50 membrane to a final volume of 1 - 2 ml.

X. COUPLING OF IgG TO SEPHAROSE 4 B

Purified IgG was covalently coupled to cyanogen bromide activated Sepharose 4 B for the purpose of affinity chromatography (60). 10 - 15 ml of packed sepharose 4 B (Pharmacia Fine Chemicals) was washed three times with an equal volume of cold distilled water and recovered by centrifugation for 5 minutes each time at 7,000 rpm. 10 ml of this packed sepharose 4 B was suspended in an equal volume of cold distilled water by means of a small magnetic stirrer. This was then transferred to a well ventillated hood containing a pH meter to which a thermometer was fitted. 4 gm. of finely divided cyanogen bromide, CNBr (Eastman Organic) were quickly added to the sepharose 4 B suspension, which was kept at a temperature of about 20° C by the addition of small pieces of ice and the pH immediately brought

to and maintained at 11.0 by the dropwise addition of 10% NaOH. Initially there was a rapid change in pH but after 8 - 10 minutes the pH changed slowly indicating a gradual cessation of proton release. At this point a large amount of ice was quickly added to the suspension and immediately transferred to a Buchner funnel and washed under suction with 10 - 15 volumes of cold 0.2 M citrate buffer pH 6.5 (61). Care was taken so that no unreacted cyanogen bromide crystals remained on the beads. Immediately one half of the activated beads was mixed with 9 - 12 mg of partially purified IgG in 5 ml of coupling buffer (0.2 M citrate pH 6.5) and stirred with a magnetic stirrer. The entire procedure of washing, addition of activated beads to IgG solution and stirring took less than 90 seconds. The slurry was kept stirring gently for 12 - 14 hours at 4° C after which time the IgG was covalently coupled to the sepharose 4 B. To determine the quantity of IgG coupled to the beads the slurry was filtered again on a Buchner funnel and washed with two or three volumes of cold coupling buffer. The beads were then resuspended in PBS pH 7.5 and ready for use. The final washings were pooled and concentrated in an Amicon chamber fitted with XM 50 membrane. The protein concentration was then determined by the Lowry method. This thus gave an indication of the quantity of ligand that was not bound to the sepharose 4 B beads.

XI. AFFINITY CHROMATOGRAPY ON ANTIGENIC FRACTIONS

(a) SOLUBILIZATION OF ANTIGENIC MATERIAL:

Stripped microsomal membrane (Band A III) prepared earlier was used as the source of antigen. This Band was first dialyzed against TKM (50 mM Tris - HCl, 50 mM KCl and 5 mM $MgCl_2$) pH 7.5 and adjusted to a protein concentration of 3 - 5 mg / ml. Detergents (DOC, Triton X 100 or SDS (Sodium Dodecyl Sulfate) were added to a final concentration of 0.5 or 1% for DOC, 1% for Triton X 100 (63) and 1% SDS and the membranes incubated for 30 minutes at room temperature. After incubation insoluble particles were removed by centrifugation for 1 hour at 100,000 X g and 4^{o} C. The solubilized membrane material was dialyzed for 24 hours against 3 or 4 changes of PBS pH 7.5. SDS solubilized membrane was first dialyzed for 12 hours against 0.2 M NaCl containing Dowex 50 H^{+} (1gm Dowex / litre of solution) then 12 hours against PBS pH 7.5.

Microsomal membrane suspensions were also sonicated in an ice bath using a Branson sonicator (Heat Systems Inc.) equipped with a microtip. Pulses of 10 seconds were given with an intensity reading of 35% followed by 20 seconds interval for cooling. The total time for sonication was 30 seconds. Soluble and sedimentable fractions were obtained by centrifugation as before. In most cases sonication was followed by DOC or Triton X 100 treatment as

above then centrifugation to remove insoluble material. Solubilization of sonicated membranes yielded in all cases only a small pellet compared to that of solubilized unsonicated membranes.

1 M KCl extraction of sonicated membranes were also carried out. The purpose for carrying out all these different method of solubilization was to decide on an efficient solubilizing agent which did not render the antigen inactive and did not cause unspecific immunoprecipitation with antiserum. Microsomal membranes suspended in 1 M KCl at concentration of 3 - 5 mg / ml were sonicated as before and incubated at room temperature for 30 minutes. The sonicate was dialyzed against 1 M KCl for 16 hours at 4° C after which time the insoluble material was separated by centrifugation for 1 hour at 100,000 X g and 4° C. Immunological tests were then carried out on the membrane fraction solubilized by the different methods described. Sonication followed by 1% Triton X 100 solubilization was found to give the best results (63). This was the method of solubilization utilized to solubilize all microsomal membrane in order to prepare the antigens for affinity chromatography.

XII. ISOLATION OF ANTIGEN(S) FROM SOLUBILIZED FRACTION

The antigens were isolated on affinity columns of Sepharose 4 B - IgG. For the preparation of affinity columns, usually 5 ml. of packed sepharose 4 B to which IgG had been coupled was used. Sepharose 4 B - IgG was packed in columns (made from sterile Plastipak syringes from which the suction cap and holder was removed) and washed with 2 - 3 column volume of PBS pH 7.5. This entire procedure was carried out in a cold room at 4^{o} C. After the washing was completed, protein concentration was determined on each of these eluted fraction (1.5 ml / fraction) and the solubilized antigen (7 - 9 mg) applied onto the column. The flow was then occluded for 1 hour, after which the column was washed with 10 column volumes (50 ml) of PBS pH 7.5 and 3 ml fraction collected (1 ml / 10 minutes). The protein concentration of each fraction was again checked to make sure that all the non - specifically bound proteins were eluted from the column. At this point the flow was stopped and a chatropic agent, 3 M potassium thiocynate KSCN, was applied to the column (64). The flow rate was adjusted to give five drops every 2 minutes and 20 drop fractions were collected manually. The protein concentration of each fraction was determined at 280 nm. It was observed that in each case the eluted antigen moved with the eluant front and was located in the first few fractions containing KSCN.

The eluted protein was dialyzed against PBS pH 7.5 and concentrated under vaccum. Because of difficulty encountered in trying to concentrate small quantities of low molecular weight antigen present in low concentration it was decided that concentrating with vaccum should be adequate.

For this process solutions containing antigens were placed in conical plastic tubes with flip-top caps (Brinkmann Laboratory) and frozen at - 20° C. Holes were punched on the caps of these tubes with a needle and placed in a dessicator containing drierite and / or anhydrous calcium chloride. A slight vaccum was applied and the dessicator was stored at 4° C until the desired volume was reached in the tubes. The tubes were then removed and stored frozen until further use.

XIII. ISOLATION OF ANTIGENS FROM ANTIGEN - ANTIBODY COMPLEXES

Negative serum (2 ml) determined by cytoplasmic immunofluorescence was brought to 50% ammonium sulfate by the addition of cold saturated ammonium sulfate and treated as in the case of positive serum. After dialysis of the precipitated protein against physiological saline (0.15 M NaCl), the solution was diluted 1 : 4 with 0.2 M Glycine - HCl pH 2.2 and applied onto a Sephadex G 200 column which had been pre-equilibrated. For equilibration, 5 gm. of medium Sephadex G 200 (Pharmacia Fine Chemicals)

were suspended in 250 ml of 0.1 M KCl and allowed to swell overnight at 4° C. The potassium chloride solution was then decanted and fresh 0.1 M KCl solution added. Equilibration was continued for another 6 - 8 hours after which time the KCl solution poured off and the swollen beads gently resuspended in 0.2 M Glycine - HCl pH 2.2 and kept at 4° C in this solution overnight after which it was ready for use.

The column (1.5 cm X 30 cm) packed to a height of about 25 cm was then eluted with 0.2 M Glycine - HCl pH 2.2 and 2 ml fractions collected at a flow rate of 1 ml every 10 minutes (1 ml / 10 minutes). Elution was carried out for 20 hours and each fraction dialyzed against PBS pH 7.5. Protein concentration was determined on each fraction. The immunological activity of each fraction was also checked against positive autologous serum and anti-human IgG. The electrophoretic pattern of the immunologically active fraction was also observed.

XIV. IMMUNOLOGICAL CHARACTERIZATION OF ANTIGENS

(a) MICROPRECIPITIN: Antigens to be tested by this method were suspended in 0.15 M NaCl. The serum used was first centrifuged for 10 minutes in a clinical centrifuge (Brinkmann, Eppendorf 3200). The clarified serum was carefully removed from the tube without disturbing the

the top lipid layer. 10 ul of this serum was placed in a capillary tube by means of a 10 ul syringe (Eppendorf) equipped with appropriate microtip and to it 10 ul of antigen was added. The solutions were mixed by gently tilting the capillary tubes back and forth at 90°. These were then left at room temperature for 1 hour or longer. A control was prepared using 0.15 M NaCl instead of the antigen. Immunologically active antigens gave rise to the formation of fluocculent precipitates whereas fractions that were inactive remained clear.

(b) OUCHTERLONY (DOUBLE - DIFFUSION PRECIPITATION):

In this method, antigen and antibody placed in wells cut in agar gel, diffused towards each other and precipitate to form an opaque line in the region where they meet in optimal proportions. 1% agar gels in 0.15 M NaCl were prepared on microscope slides (65) fitted onto standard gel making apparatus (Gelman Instruments Co.). The gels were allowed to set for 30 minutes after which, wells were punched with a commercially available (Gelman) gel cutter. Serum, clarified as before, and antigens were inserted in the appropriate wells and kept in a moist chamber for 24 hours or longer if necessary, at room temperature for the diffusion and precipitation process to take place. The precipitation lines usually appeared within 24 hours and clearly visible against a suitable background under favourable lighting conditions. The agar plates were kept

for an additional 24 hours after the appearance of the precipitation lines to facilitate optimal development of precipitation lines. When the diffusion and precipitation process were considered to be completed, the gels were removed from the moist chamber dried, stained, labelled and stored.

(i) DRYING: To dry the gel each plate was covered with a 3 X 10 cm sheet of smooth water - wetted and blotted filter paper and placed in a 37° C incubator for 12 - 14 hours (66). When thoroughly dried the filter paper detached itself from the glass and was easily lifted from the dried agar.

(ii)STAINING: The plates were washed under a gentle stream of running water to remove any residual filter paper fibres. The agar plates were then transferred to a bath containing the staining solution consisting of 0.2% Ponceau S or 0.5% Amido black in 3% trichloroacetic acid (67) for 5 - 10 minutes at which time the precipitation lines intensified and were clearly visible. The plates were removed and placed in a solution of 5% aqueous acetic acid and 5% glycerol to wash off the excess stain and clear the background. The washing solution was changed a few times until the background became clear and the bath colourless. The plates were dried at room temperature labelled and stored.

(c) COUNTERCURRENT IMMUNOELECTROPHORESIS (CROSS - OVER ELECTROPHORESIS):

This technique, which combines electrophoresis with immunoprecipitation, is a very ingenious and elegant method for demonstrating the precipitation reaction. It is based on the fact that due to endosmotic flow antibodies, being gamma globulins, move towards the cathode in an electrical field, whereas antigenic substances have a different electrophoretic mobility and migrate towards the anode. By placing the antibody containing specimen on the anodic side and the antigen on the cathodic side, of the appropriate gels both reactants can be made to migrate towards each other forming a precipitation line between the sites of application. This technique maintains the specificity characteristic of the Ouchterlony gel diffusion method, yet detects in 1 to 2 hours one - tenth the amount of antigen required for gel diffusion (68, 69).

PREPARATION OF AGAROSE CELS: To prepare gel, 0.5 gm. of Agarose (Sigma Type II Medium EEO) was dissolved in 50 ml of Baribitone buffer (prepared with 5 gm of Sodium diethylbarbiturate $NaC_8H_{12}N_2O_3$ (Fisher Scientific), 3.33 gm of sodium acetate $Na\ CH_3\ COO^-.\ 3H_2O$, 1 gm NaN_3 in 1 litre of distilled water; pH adjusted with HCl) pH 8.2, ionic strength 0.05, by bringing to a boil on a hot plate (70). 25 ml aliquots were pipetted into precleaned plates purchased from Hyland (Travenol Laboratories Inc.) making

sure that the gel was evenly layered with the tip of the pipette. The gels were covered and allowed to remain undisturbed for a minimum of 1 hour to set (71).

All antigenic fraction to be tested were dialyzed in a cold room at 4° C against 0.15 M NaCl prior to use and kept in small tubes for at least 30 minutes at room temperature to equilibrate. Antigen at a concentration greater than 0.5 mg / ml were diluted to 1 : 4 or 1 : 8. Antigen less than 0.5 mg / ml were diluted 1 : 1 with Barbitone buffer. The serum or IgG fractions used were diluted 1 : 16 with 0.2 M Glycine — HCl pH 2.2. For very low antigen concentration (less than 0.5 mg / ml or 10 ug / 20 ul), the antibody was diluted 1 : 32 with the same buffer and also kept at room temperature. Controls such as anti-human IgG and normal saline were treated similarly.

After the gel had set square wells were cut on the agarose gel. Wells were cut in pairs with a distance of 0.5 cm between opposing vertical edges of each pair of wells and 1 cm between opposing horizontal edges of adjacent pairs. A maximum of 21 pairs of wells can be cut on a gel of this size (8 X 13 X 0.24 cm). The cut gel was removed from each well by suction. 20 ul samples were then applied to each well. This quantity was found to give best results as it facilitated maximum electrical conduction

through the well.

After application of the samples the plate was inverted so that the wells of each pair containing the acidified serum or IgG will be near the anode when mounted on the chamber. Immunoelectrophoresis was carried out using an Hyland Immunoelectrophoresis apparatus (Hyland Division, Travenol Laboratory Inc.) equipped with electrophoretic chamber and built in power supply. The electrode buffer was same as that used to prepare the gel (Barbitone / Veronal pH 8.2). A running time of 1 hour at a current of 30 mAmps was sufficient for precipitation line to form. The precipitation lines were most clearly seen under a UV light source with a black background. The gel was easily removed from the plate with a thin spatula and transferred onto a glass plate where it was dried and stained by the same technique previously described for Ouchterlony plates.

XV. BIOCHEMICAL CHARACTERIZATION OF FRACTIONS AND ANTIGENS

(a) PREPARATION OF POLYACRYLAMIDE GELS: 5.6% gels were prepared according to the method of Fairbanks et al (72). The following solution were used to prepare the gels.

SOLUTION 1 Concentrated Acrylamide solution. 40 gm Acrylamide $CH_2CHCONH_2$ and 1.5 gm Methylene bis Acrylamide dissolved in water to 100 ml.

SOLUTION 2 20% SDS (Sodium Dodecylsulfate). 20 gm SDS in water to 100 ml (w / v).

SOLUTION 3 1.5% Ammonium Persulfate $(NH_4)_2S_2O_8$ (w / v). 15 mg Ammonium Persulfate in 1 ml water.

SOLUTION 4 0.5% N,N,N'N' Tetramethylethylenediamine (TEMED) (v / v). 5 ul TEMED in 1 ml water.

SOLUTION 5 Overlaying solution. 0.025 ml of 20% SDS, 0.5 ml of 1.5% Ammonium Persulfate and 0.5 ml of 0.5% TEMED. Bring to 5 ml with water.

SOLUTION 6 Buffer 10 X concentrated pH 7.4 80 ml of 1.0 M Tris - hydroxymethylaminomethane (Tris), 20 ml of 2.0 M Sodium Acetate (anhydrous) $CH_3COO^-Na^+$, 20 ml of 0.2 M Ethylediamine Tetraacetic Acid (Na_2EDTA) and adjust to pH 7.4 with Acetic Acid. Then bring to 200 ml with water.

To prepare 10 ml of gel solution, enough to make 3 gels (3 ml per glass tube 6 mm diameter and 13 cm long), the following quantities of the above solution were mixed.

SOLUTION 1	1.4 ml
SOLUTION 2	0.5 ml
SOLUTION 3	1.0 ml
SOLUTION 4	0.5 ml
SOLUTION 6	1.0 ml
WATER	5.6 ml
TOTAL	10.0 ml

Solution 1, 2, 6 and water were mixed in the above quantities and deaerated for 15 - 30 minutes. Solutions 3 and 4 were then added to the deaerated solutions, mixed and added to cleaned gel tubes. Each tube was then overlaid with solution 5 and allowed to polymerize at room temperature. After polymerization was completed (about 45 minutes) the overlaying solution was removed, the top of the gel rinsed with 1 : 10 diluted solution 6, overlaid with same buffer solution and left to set overnight.

(b) PREPARATION OF SAMPLES ~~FOR~~ GEL ELECTROPHORESIS:

The following stock solutions were prepared for use.

SOLUTION 1	50% sucrose
SOLUTION 2	10 mM EDTA, 100 mM Tris - HCl pH 8
SOLUTION 3	400 mM Dithiothreitol (DTT)

All the above solutions were prepared 10 X concentrated.

Each sample containing about 100 ug of protein was first dialyzed for 12 - 14 hours in a cold room at 4° C against a buffer made from 1 mM DTT, 1 mM EDTA and 10 mM Tris - HCl pH 8. To each sample was then added the following to the stated final concentration: 1% SDS, 5% Sucrose, 10 mM EDTA, and 40 mM DTT using the stock solutions prepared. The samples were incubated for 25 minutes at 37° C. 5 - 10 ul of Tracking Dye (Bromophenol Blue, Sigma) were added to each tube mixed and ready for use. Control samples viz. Ribonuclease A, Chymotrypsinogen A, Ovalbumin, Aldolase and Bovine Serum Albumin were treated similarly.

XVI. ELECTROPHORESIS AND STAINING OF GELS: Protein samples were applied on previously prepared 5.6% gels fitted onto a locally made gel chamber attached to an Isco power supply. The electrophoresis buffer was prepared by mixing 100 ml of solution 6 (buffer 10 X concentrated pH 7.4), 50 ml of solution 2 (20% SDS) and brought to a volume of 1 litre with 850 ml of water. A current of 8 mAmps / tube was applied and the length of time of each run was between 4 - 5 hours. When the run was completed the gels were measured, removed from the gel tube with the aid of a syringe and needle, stained and destained.

For staining and destaining gels the following solutions were prepared.

SOLUTION 1 Fixing Solution: 125 ml of Isopropyl Alcohol (Reagent Grade) 25%, 50 ml of Acetic Acid (Glacial) 10%, 250 mg Coomassie Brilliant Blue R (Sigma) 0.05%, brought to 500 ml with water and filtered to remove undissolved dye.

SOLUTION 2 Staining solution: (a) 50 ml of Isopropyl Alcohol 10%, 50 ml of Acetic Acid 10%, 25 mg Coomassie Brilliant Blue R 0.005%, brought to 500 ml with water and filtered to remove undissolved dye.

SOLUTION 3 Staining solution: (b) 50 ml of Acetic Acid 10%, 12.5 mg Coomassie Brilliant Blue R 0.0025%, brought to 500 ml with water and filtered to remove undissolved dye.

SOLUTION 4 Destaining solution: 100 ml of Acetic Acid in 1 litre of water.

Gels were stained for 12 - 15 hours in solution 1 then 6 - 9 hours in solution 2. Staining in solution 3 was done for the purpose of intensifying the protein bands. The gels were kept for another 12 - 15 hours in this

solution before destaining with solution 4 until the background was clear. At this stage the protein bands and gel lengths were measured.

XVII. SLICING OF GELS; Gels to be sliced were always prepared and electrophoresed in duplicate. One gel was stained and the other frozen for slicing. The gel was mounted unto an automatic Joyce Loebl gel slicer, held firmly with a solution of 10% glycerol which is frozen on the gel by finely divided dry ice and cut into 1 mm thick slices. Slices were transferred to small tubes to which 0.5 ml quantity of 0.2 M NaCl were added. The gel was ground up with a small loose fitting homogenizer and incubated at 0° C for 6 hours. The suspensions were centrifuged for 10 minutes at 3,000 rpm and 4° C. The supernatants were removed and dialyzed at room temperature for 16 hours against 0.2 M NaCl solution containing Dowex 50 H^{+} (1 gm Dowex / litre solution) (Dowex 50 W - Hydrogen, Strongly Acid Cation, Sigma). These fractions were then tested for immunological activity using the methods described previously.

XVIII. CHEMICAL CHARACTERIZATION OF FRACTIONS AND ANTIGENS

(a) PROTEIN DETERMINATION: For this procedure the following solutions were freshly prepared each time (47).

SOLUTION 1	2% Na_2CO_3 in 0.1 N NaOH
SOLUTION 2	0.5% $CuSO_4.5\ H_2O$ in 1% Sodium Tartrate
SOLUTION 3	50 ml solution 1 : 1 ml solution 2
SOLUTION 4	1 volume Folin - Ciocolteau Reagent: 2.5 volumes water (Fisher Scientific).

The assay mixture consisted a total of 3.9 ml. This was made up using the prepared solutions, water and protein in the following quantity. To each assay tube was added 0.5 ml of water from which was removed the exact quantity as the volume of protein solution to be added. The protein solution then added and to each tube 3 ml of solution 3 was added, mixed gently and allowed to stand 10 minutes at room temperature. Then 0.3 ml of solution 4 was rapidly added, stirred immediately with a vortex and kept for 30 minutes at room temperature in the dark to facilitate the formation of the biuret complex and the reduction of the phosphomolybdate and phosphotungstate, which are in the solution 4 added, by the tyrosine, tryptophan and cysteine residues in the protein molecule. Standards were prepared each time using a stock solution

of 1 mg / ml Bovine Serum Albumin (BSA) in the range of 0 - 100 ug protein. The absorbance was then read at 600 nm in the spectrophotometer. From the standard curve plotted the protein concentrations of the unknown solutions were extrapolated.

(b) RNA (RIBONUCLEIC ACID) DETERMINATION: For this assay a solution containing 0.2 gm of Orcinol in 20 ml of concentrated HCl containing 0.1 gm $FeCl_3$ was prepared each time (46). 2 ml of water was added to each tube and from this was removed the exact quantity as the volume of RNA solution to be added (RNA solutions were thoroughly dialyzed against 0.15 M NaCl). To this was added 2.0 ml of Orcinol solution, shaken gently and placed in a 100° C water bath for 20 minutes to facilitate the formation of the as yet unknown coloured complex of Orcinol, Fe^{3+} and ribose. Standards were prepared each time using a stock solution of 1 mg / ml ribonucleic acid in the range 0 - 100 mg RNA. The absorbance was read at 660 nm. in the spectrophotometer. From the standard curve plotted the RNA concentration of the unknown solution were extrapolated.

(c) PHOSPHOLIPID DETERMINATION: Total lipid extraction was carried out according to the method of Folch et al (74). For this process 0.5 ml of each sample suspended in 0.15 M NaCl was transferred to a separatory funnel containing 15 ml of 2 : 1 chloroform - methanol (v/v)

and shaken vigorously. In some cases the sample was homogenized with a loose - fitting homogenizer before transferring to the separatory funnel. After extraction, the chloroform - methanol mixture was filtered through pre-washed glass wool in a 50 ml round bottom flask and the chloroform - methanol removed under vaccum. The dry lipid extract was then successively washed once with a mixture of 10 ml of 2 : 1 chloroform - methanol and 2 ml of 0.15 M NaCl then twice with a mixture 5 ml of 2 : 1 chloroform - methanol and 1 ml of 0.15 M NaCl. Each washing was transferred to a separatory funnel and again vigorously shaken. The mixture then allowed to separate into two distinct phases after which the top phase was removed by suction. The lower phase was transferred to a round bottom flask and the solution removed under vaccum. To the dry lipid extract a small quantity of absolute alcohol was added to remove traces of water and this was subsequently removed by vaccum. The dry lipid was then redissolved in a minimum amount of chloroform - methanol (usually 0.5 ml) and all or portion of it was used for phosphorus analysis.

For Band III and Band A III 0.05 ml of the lipid extract was used for hydrolysis whereas for all other fractions 0.50 ml was used. Each sample was transferred to a 13 X 100 mm Pyrex test tube and taken to dryness under

a N_2 stream (75). Then 0.3 ml of 1 N $HClO_4$ was added and the tubes heated in a boiling water bath for 15 minutes to hydrolyze the phosphate esters of the phospholipids. The samples were then analyzed for inorganic phosphate as done previously except only 0.1 ml of phosphorous sample was used. For calculation of phospholipid content it was assumed that 25 mg of phospholipid contain 1 mg phospholipid phosphorus (76).

XIX. MORPHOLOGICAL CHARACTERIZATION OF FRACTIONS

(a) ELECTRON MICROSCOPY: All electron microscopy were done at the Mc Gill University Cancer Unit, microscopy department. Microsome samples suspended in 0.25 M sucrose were fixed by the addition of 1 volume of 4% glutaraldehyde in 0.2 M sodium cacodylate pH 7.2 and kept at 0° C for 60 minutes. The microsomes were then centrifuged at 100,000 X g for 30 minutes and the resulting pellet post-fixed for 2 hours in the cold with 2% OsO_4 in 0.1 M sodium cacodylate pH 7.2. Further fixing, staining, dehydrating, embedding, sectioning and photographing were done by the departmental electron microscopist.

RESULTS

TABLE 1

CLINICAL HISTORY OF PATIENTS: In this project 10 malignant melanoma patients tissue were studied.

NAME OF PATIENT	DATE OF SURGERY	ORGAN OR TISSUE USED
FEI	December 6,1976	Right and left lymph nodes
MOR	December 13,1976	Neck lymph node
OLI	August 14,1975	Axilla
PER	December 3,1974	Left arm
ROS	May 5,1975	Right and left axillary lymph node
SHU	August 18,1975	Lymph node
TRE	February 20,1976	Right and left axilla
VEN	January 15,1975	Lung
ZAI	October 28,1976	Right lymph node
ELL	July 15,1977	Lymph node

A tissue culture melanoma cell line was also used as a control.

IgR	Sub 55	September 9,1976	Tissue culture

TABLE 2

DETERMINATION OF MELANOMA SPECIFIC POSITIVE ANTIBODY BY CYTOPLASMIC IMMUNOFLUORESCENE: (+) indicates high titre, ($\pm$) indicates low titre and (-) indicates no melanoma specific antibody.

PATIENT NAME	DATES OF SERUM USED	IMMUNOFLUORESCENE PATTERN
FEI	6 - 12 - 76	+
	3 - 2 - 77	+
	19 - 1 - 77	$\pm$
	26 - 1 - 77	-
MOR	23 - 3 - 77	+
	13 - 7 - 77	$\pm$
	10 - 8 - 77	$\pm$
	17 - 8 - 77	$\pm$
	27 - 7 - 77	$\pm$
OLI	30 - 7 - 75	$\pm$
	2 - 4 - 75	$\pm$
	18 - 8 - 75	$\pm$
PER	4 - 10 - 74	+
	4 - 9 - 74	$\pm$
	11 - 9 - 74	-
	30 - 10 - 74	-

TABLE 2 CONTINUED

ROS	21 - 8 - 75	+
	3 - 9 - 75	+
	13 - 8 - 75	+
	8 - 7 - 75	±
SHU	28 - 4 - 76	+
	31 - 3 - 76	+
	30 - 6 - 76	+
	4 - 5 - 77	±
TRE	6 - 4 - 77	+
	26 - 1 - 77	+
	16 - 3 - 77	+
	1 - 6 - 77	+
VEN	13 - 8 - 75	+
	20 - 8 - 75	±
	18 - 9 - 75	±
	23 - 9 - 75	-
ZAI	2 - 11 - 76	+
	28 - 10 - 76	+
	15 - 2 - 77	+
	9 - 2 - 77	±

TABLE 2 CONTINUED

ELL	13 - 7 - 77	+
	27 - 7 - 77	+
	10 - 8 - 77	+

Serum from two normal patients, DiM and CHE dated 21 - 10 - 75 and from ROO, 8 - 9 - 77, a teratoma patient and CHA, 22 - 6 - 77 a liposarcoma were also used for control experiments.

TABLE 3

TITRE OF POSITIVE ANTIBODY AGAINST AUTOLOGOUS CYTOPLASMIC SMEARS

PATIENT SERUM	DILUTION FACTOR					
	1/4	1/8	1/16	1/32	1/64	1/128
MOR 23- 3-77	+	+	+	+	+	+
OLI 30- 7-75	+	+	+	+	±	±
PER 4-10-74	+	+	+	+	±	±
TRE 6- 4-77	+	+	+	+	±	±
VEN 13- 8-75	+	+	+	+	+	±
NEGATIVE SERUM	±	-	-	-	-	-

TABLE 4

TITRE OF POSITIVE ANTIBODY AGAINST AUTOLOGOUS AND ALLOGENEIC CYTOPLASMIC SMEARS

PATIENT	SERUM	CORRESPONDING PATIENT'S SMEARS				
		FEI	ROS	SHU	ZAI	ELL
FEI	6-12-76	1/128	1/ 64	1/128	1/ 32	1/ 64
	3- 2-77	1/ 64	1/ 64	1/ 64	1/ 32	1/ 32
ROS	21- 8-75	1/ 32	1/ 64	1/ 64	1/ 32	1/ 32
	3- 9-75	1/128	1/128	1/ 64	1/ 64	1/ 64
SHU	28- 4-76	1/ 64	1/128	1/128	1/ 64	1/ 32
	31- 3-76	1/ 32	1/ 64	1/ 64	1/ 32	1/ 64
ZAI	2-11-76	1/ 64	1/ 32	1/ 32	1/ 64	1/ 64
	28-10-76	1/ 64	1/ 32	1/ 64	1/ 32	1/ 64
ELL	13- 7-77	1/ 32	1/ 64	1/ 64	1/ 32	1/ 64
	27- 7-77	1/ 64	1/ 64	1/128	1/ 64	1/128

Each serum was diluted 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 with PBS (0.01 M potassium phosphate and 0.15 M NaCl) pH 7.3. The sera with the highest titre were used for further work.

FIG. 1. PROTEIN STANDARD CURVE.

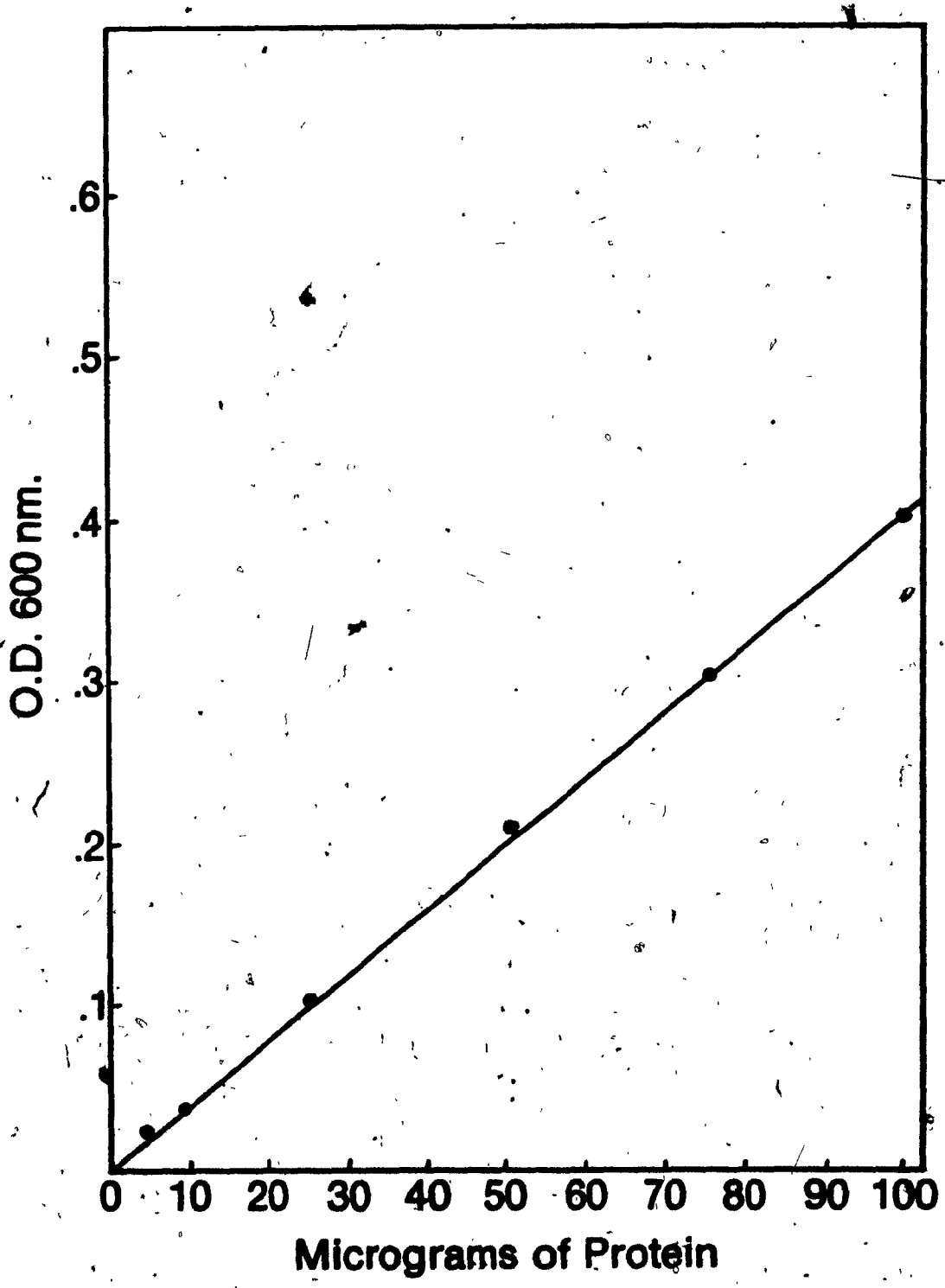


TABLE 5 (a)

LOCALIZATION OF IMMUNOLOGICALLY ACTIVE FRACTION FROM FRACTIONATED AND SUBFRACTIONATED CELLS:

Immunological activity tabulated as (+) for strong immunoprecipitation, (±) for weak activity and (-) for no reaction. Each fraction was tested against autologous positive serum by Ouchterlony and countercurrent immunoelectrophoresis. The immunological activity indicated is that from immunoelectrophoresis on 1% agarose gels.

PATIENT 1 NAME : PER

FRACTION TESTED	TOTAL VOLUME	PROTEIN CONCENTRATIONS		IMMUNOLOGICAL TEST	
		O.D. at 600 nm.	PROTEIN EQUIV. mg / ml	DILUTIONS	ACTIVITY
	ml				
HOMOGENATE	16	0.308/ 5ul	15.2	1/32	+
PELLET 1	10	0.175/ 5ul	8.1	1/16	-
PELLET 2	5	0.188/10ul	4.5	1/ 8	±
PELLET 3	5	0.145/10ul	3.4	1/ 8	-
BAND I	3	0.055/10ul	1.1	1/ 4	±
BAND II	3	0.240/10ul	5.8	1/16	-
BAND III	3	0.218/10ul	5.3	1/16	+
BAND A I	1	0.207/10ul	4.9	1/16	-
BAND A II	1	0.192/10ul	4.7	1/16	-
BAND A III	1	0.276/10ul	7.0	1/16	+
FRACTION I	1	0.245/10ul	5.9	1/16	+
FRACTION II	0.5	0.015/20ul	0.15	1/ 4	+

TABLE 5 (b)

LOCALIZATION OF IMMULOGICALLY ACTIVE FRACTION FROM FRACTIONATED AND SUBFRACTIONATED CELLS:

Comparison for similarity in immunologically active fractions between the previous patient and fractions obtained from this patient.

PATIENT 2 NAME : VEN

FRACTION TESTED*	TOTAL VOLUME	PROTEIN CONCENTRATIONS		IMMUNOLOGICAL TEST	
		O.D. at 600 nm.	PROTEIN EQUIV. mg / ml	DILUTIONS	ACTIVITY
	ml				
HOMOGENATE	12	0.242/ 5ul	11.8	1/32	+
PELLET 1	8	0.138/10ul	6.4	1/16	-
PELLET 2	5	0.146/10ul	3.5	1/ 8	±
PELLET 3	5	0.115/10ul	2.8	1/ 8	-
BAND I	3	0.044/10ul	0.98	1/ 4	±
BAND II	3	0.216/10ul	5.0	1/16	-
BAND III	3	0.185/10ul	4.4	1/16	+
BAND A 1	1	0.168/10ul	4.0	1/16	-
BAND A 11	1	0.152/10ul	3.6	1/ 8	-
BAND A 111	1	0.225/10ul	5.4	1/16	+
FRACTION I	1	0.204/10ul	4.9	1/16	+
FRACTION II	0.5	0.013/20ul	0.13	1/ 4	+

* Each fraction was tested by immunoelectrophoresis using autologous positive serum.

TABLE 5 (c)

LOCALIZATION OF IMMUNOLOGICALLY ACTIVE FRACTION FROM FRACTIONATED AND SUBFRACTIONATED CELLS:

Comparison for similarity in immunologically active fractions between the previous two patients and fractions obtained from this patient.

PATIENT 3 NAME : MOR

FRACTION TESTED*	TOTAL VOLUME	PROTEIN CONCENTRATIONS		IMMUNOLOGICAL TEST	
		O.D. at 600 nm.	PROTEIN EQUIV. mg / ml	DILUTIONS	ACTIVITY
HOMOGENATE	12	0.282/ 5ul	13.8	1/32	+
PELLET 1	8	0.155/ 5ul	7.2	1/16	-
PELLET 2	5	0.158/10ul	3.7	1/ 8	-
PELLET 3	5	0.125/10ul	2.9	1/ 8	-
BAND I	3	0.056/10ul	1.1	1/ 4	+ -
BAND II	3	0.238/10ul	5.7	1/16	-
BAND III	3	0.195/10ul	4.8	1/16	+
BAND A I	1	0.170/10ul	3.4	1/ 8	-
BAND A II	1	0.163/10ul	3.9	1/ 8	-
BAND A III	1	0.214/10ul	4.9	1/16	+
FRACTION I	1	0.178/10ul	4.5	1/16	+
FRACTION II	o.5	0.020/20ul	0.2	1/ 4	+

* Each fraction was tested by immunoelectrophoresis using autologous positive serum.

FIG. 2 RNA STANDARD CURVE.

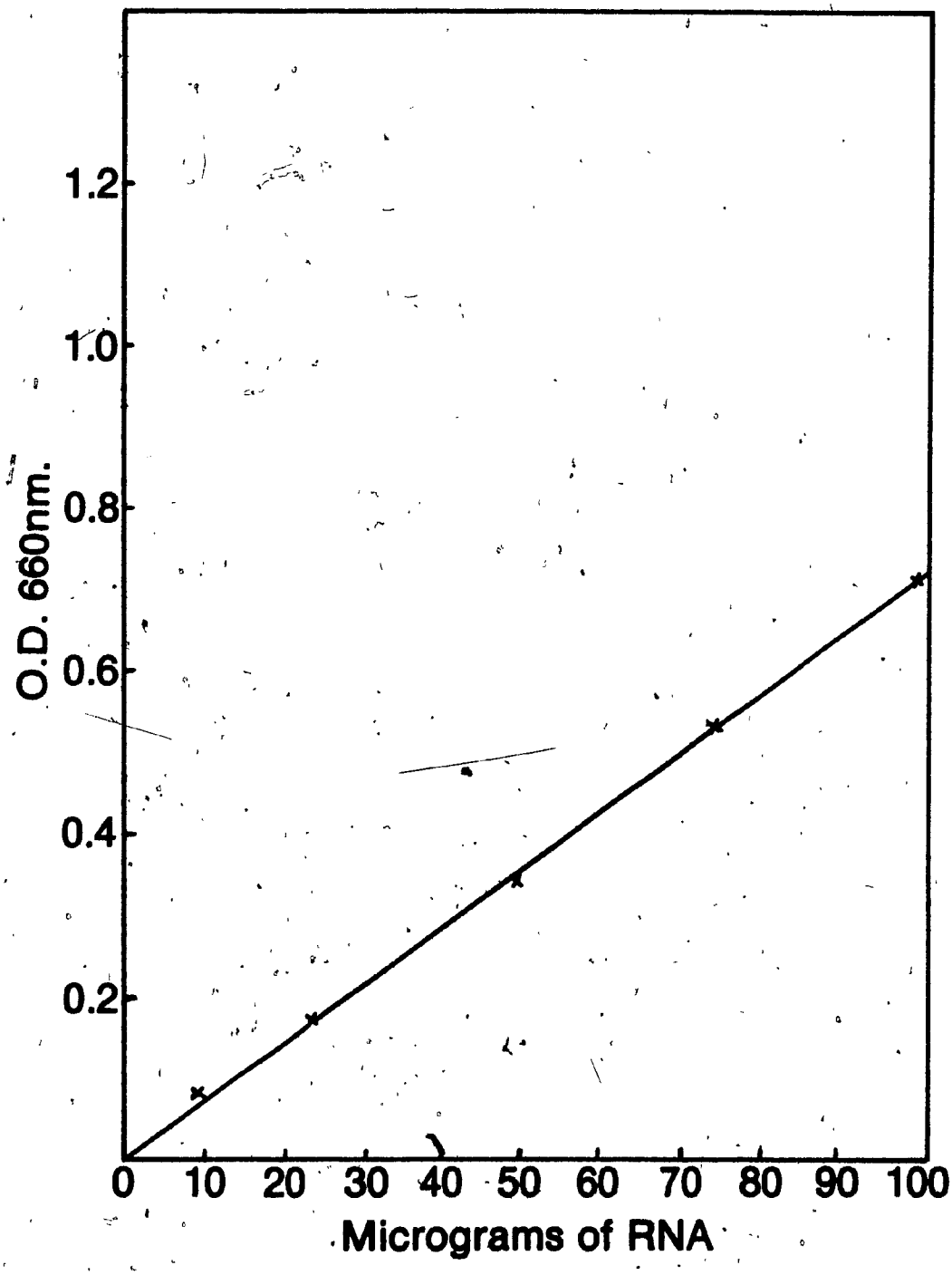


FIG. 3 PHOSPHORUS STANDARD CURVE.

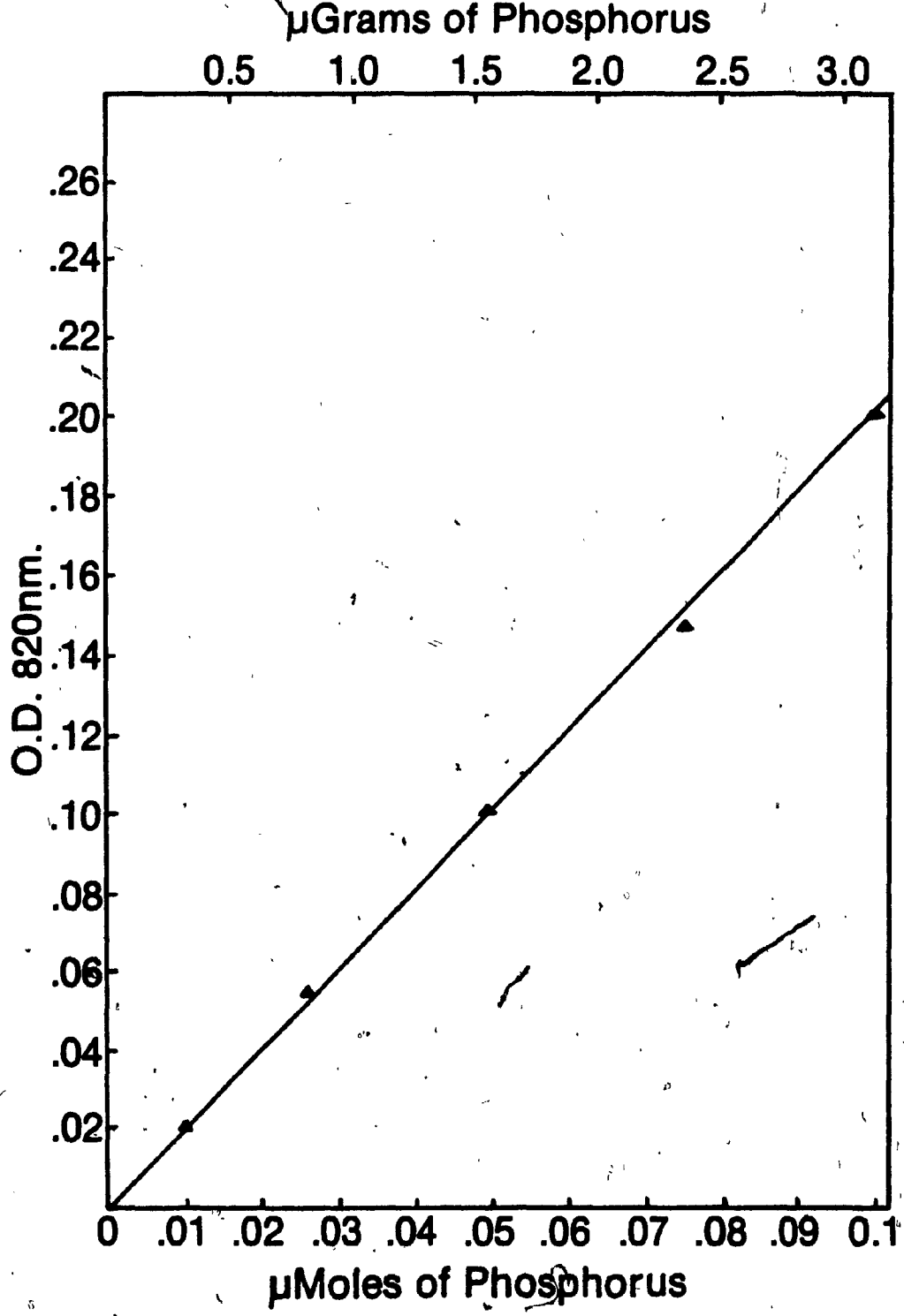


TABLE 6 (a)

RIBONUCLEIC ACID AND PHOSPHOLIPID ANALYSIS ON ROUGH MICROSOMES AND STRIPPED ROUGH MICROSOMES FROM THREE PATIENTS PER, VEN AND MOR.

FRACTION DESCRIPTION	RIBONUCLEIC ACID		PHOSPHOLIPID*	
	O.D. 660 nm.	RNA EQUIV.	O.D. 820 nm.	PLP EQUIV.
PER BAND III	0.621	90 ug/50ul	0.230	10.8ug/ 50ul
VEN BAND III	0.520	75 ug/50ul	0.180	8.4ug/ 50ul
MOR BAND III	0.555	80 ug/50ul	0.185	8.6ug/ 50ul
PER BAND A I	0.555	80 ug/25ul	0.155	7.2ug/500ul
VEN BAND A I	0.435	62.5ug/25ul	0.120	5.6ug/500ul
MOR BAND A I	0.365	52.5ug/25ul	0.105	5.0ug/500ul
PER BAND A II	0.500	72.5ug/25ul	0.145	6.8ug/500ul
VEN BAND A II	0.380	55 ug/25ul	0.110	5.2ug/500ul
MOR BAND A II	0.435	62.5ug/25ul	0.120	5.6ug/500ul
PER BAND A III	0.185	26 ug/50ul	0.235	11.0ug/ 50ul
VEN BAND A III	0.160	23 ug/50ul	0.175	8.2ug/ 50ul
MOR BAND A III	0.150	21 ug/50ul	0.155	7.2ug/ 50ul

*To obtain true values of PLP in ug from the OD 820 nm readings a factor of 3 is used since only 1/3 of original phosphorus solution was used in the assay. Also it was assumed that 25 mg of phospholipid contains 1 mg of phospholipid phosphorus.

TABLE 6 (b)

CHEMICAL ANALYSIS OF ROUGH MICROSOMES AND MICROSOMAL FRACTIONS OBTAINED BY 0.79 mM PUROMYCIN TREATMENT OF ROUGH MICROSOMES FROM THREE PATIENTS PER, VEN AND MOR.

FRACTION DESCRIPTION	PROTEIN mg / ml	RNA mg / ml	PLP mg / ml
PER BAND III*	10.2	1.8	5.4
VEN BAND III	8.3	1.5	4.2
MOR BAND III	8.8	1.6	4.3
PER BAND A I	4.9	3.2	0.36
VEN BAND A I	4.0	2.5	0.28
MOR BAND A I	3.4	2.1	0.25
PER BAND A II	4.7	2.9	0.34
VEN BAND A II	3.6	2.2	0.26
MOR BAND A II	3.9	2.5	0.28
PER BAND A III	7.0	0.52	5.5
VEN BAND A III	5.4	0.46	4.1
MOR BAND A III	4.9	0.42	3.6

*In each case 2.0 ml of rough microsomes (BAND III) at the concentrations stated previously were pelleted and resuspended in 1 ml of 0.25 M STKM (sucrose, 50 mM Tris - HCl, 750 mM KCl and 5 mM $MgCl_2$) containing puromycin. Abbr. PLP (phospholipid), RNA (ribonucleic acid).

TABLE 7 (a)

ISOLATION OF MICROSOMES FROM AN ADDITIONAL SEVEN MALIGNANT MELANOMA PATIENTS.

PATIENT	FRACTION DESCRIPTION*	TOTAL VOLUME ml	O.D. 600 nm	PROTEIN mg / ml
OLI	HOMOGENATE	12	0.252/ 5ul	12.2
	BAND I	3	0.035/10ul	0.7
	BAND II	3	0.180/10ul	4.3
	BAND III	3	0.162/10ul	3.7
TRE	HOMOGENATE	12	0.340/ 5ul	16.8
	BAND I	3	0.064/10ul	1.4
	BAND II	3	0.248/10ul	6.0
	BAND III	3	0.132/10ul	3.1
FEI	HOMOGENATE	12	0.345/ 5ul	16.8
	BAND I	3	0.058/10ul	1.3
	BAND II	3	0.290/10ul	7.1
	BAND III	3	0.222/10ul	5.3
ZAI	HOMOGENATE	16	0.476/ 5ul	23.6
	BAND I	3	0.075/10ul	1.7
	BAND II	3	0.295/10ul	7.3
	BAND III	3	0.281/10µl	6.8

TABLE 7 (a) CONTINUTED

SHU	HOMOGENATE	16	0.464/ 5ul	23.0
	BAND I	3	0.053/10ul	1.2
	BAND II	3	0.230/10ul	5.5
	BAND III	3	0.206/10ul	4.9
ROS	HOMOGENATE	16	0.560/ 5ul	28.0
	BAND I	3	0.071/10ul	1.6
	BAND II	3	0.273/10ul	5.4
	BAND III	3	0.245/10ul	6.1
ELL	HOMOGENATE	16	0.504/ 5ul	24.8
	BAND I	3	0.058/10ul	1.3
	BAND II	3	0.262/10ul	6.3
	BAND III	3	0.248/10ul	6.0
FEI‡	HOMOGENATE	16	0.300/ 5ul	14.8
	BAND I	3	0.062/10ul	1.3
	BAND II	3	0.246/10ul	6.1
	BAND III	3	0.238/10ul	5.6

*Enzyme assays were done on the above fractions from all ten patients. For patients ZAI, SHU, ROS, AND ELL between 7 - 8 gm of frozen tissue were used whereas for other patients 4 - 5 gm of tissue were available for use in rough microsomal isolation. Band I possibly contains post - golgi vesicles, Band II contains smooth endoplasmic

TABLE 7 (a) CONTINUED

reticulum (SER) and Band III contains rough endoplasmic reticulum (RER) (See Plate 3)

‡ 2nd isolation using same tissue from this patient.

TABLE 7 (b)

ISOLATION OF MICROSOMES FROM A TISSUE CULTURE MELANOMA CELL LINE

FRACTION DESCRIPTION	TOTAL VOLUME ml	O.D. 600 nm	PROTEIN mg / ml	IMMUNOLOGICAL ACTIVITY
HOMOGENATE	12	0.290/10ul	7.1	+
BAND I	3	0.025/10ul	0.6	±
BAND II	3	0.110/10ul	2.5	-
BAND III	3	0.130/10ul	3.0	+

IgR melanoma was used to standardize fractionations procedures and as control for immunological assay.

TABLE 8 (a)

DISTRIBUTION OF ATP'ase ACTIVITY ON SUBCELLULAR FRACTIONS OBTAINED FROM MICROSOMAL PREPARATION.

PATIENT	FRACTION	O.D.820 (-OUBAIN)	O.D.820 (+OUBAIN)	ΔO.D.820
PER	HOMOGENATE	0.064	0.050	0.014
	BAND I	0.033	0.030	0.003
	BAND II	0.155	0.120	0.035
	BAND III	0.068	0.045	0.023
VEN	HOMOGENATE	0.100	0.065	0.035
	BAND I	0.025	0.024	0.001
	BAND II	0.285	0.210	0.075
	BAND III	0.160	0.082	0.078
MOR	HOMOGENATE	0.080	0.048	0.032
	BAND I	0.045	0.035	0.010
	BAND II	0.235	0.172	0.063
	BAND III	0.120	0.050	0.070
OLI	HOMOGENATE	0.105	0.070	0.035
	BAND I	0.032	0.030	0.002
	BAND II	0.155	0.095	0.060
	BAND III	0.160	0.098	0.072

TABLE 8 (a) CONTINUED

TRE	HOMOGENATE	0.065	0.035	0.030
	BAND I	0.021	0.020	0.001
	BAND II	0.225	0.165	0.060
	BAND III	0.080	0.035	0.065
FEI	HOMOGENATE	0.082	0.065	0.017
	BAND I	0.032	0.031	0.001
	BAND II	0.198	0.166	0.032
	BAND III	0.065	0.035	0.030
ZAI	HOMOGENATE	0.070	0.055	0.015
	BAND I	0.041	0.040	0.001
	BAND II	0.210	0.168	0.042
	BAND III	0.071	0.042	0.029
SHU	HOMOGENATE	0.045	0.034	0.011
	BAND I	0.038	0.029	0.009
	BAND II	0.129	0.105	0.024
	BAND III	0.110	0.088	0.022
ROS	HOMOGENATE	0.112	0.085	0.027
	BAND I	0.045	0.040	0.005
	BAND II	0.185	0.132	0.053
	BAND III	0.105	0.065	0.040

TABLE 8 (a) CONTINUED

ELL	HOMOGENATE	0.091	0.073	0.018
	BAND I	0.035	0.032	0.003
	BAND II	0.215	0.175	0.040
	BAND III	0.120	10.088	0.032

TABLE 8 (b)

SPECIFIC ACTIVITY AND RELATIVE SPECIFIC ACTIVITY OF Na^+ / K^+ DEPENDENT ATP'ase.

PATIENT	FRACTION	O.D.820 nm	uMoles P_i/ 60 mins/ 50 ug PROTEIN	SPECIFIC ACTIVITY uMoles P_i/60mins/ mgPROTEIN	RELATIVE* SPECIFIC ACTIVITY
PER	HOMOGENATE	0.014	0.007	0.140	1.00
	BAND I	0.003	0.0015	0.030	0.21
	BAND II	0.035	0.0175	0.350	2.50
	BAND III	0.023	0.0115	0.230	1.64
VEN	HOMOGENATE	0.035	0.0175	0.350	1.00
	BAND I	0.001	0.0005	0.010	0.03
	BAND II	0.075	0.0375	0.750	2.14
	BAND III	0.078	0.0390	0.780	2.22
MOR	HOMOGENATE	0.032	0.0160	0.320	1.00
	BAND I	0.010	0.0050	0.100	0.31

TABLE 8 (b) CONTINUED

	BAND II	0.063	0.0315	0.630	1.97
	BAND III	0.070	0.0350	0.700	2.19
OLI	HOMOGENATE	0.035	0.0175	0.350	1.00
	BAND I	0.002	0.0010	0.020	0.06
	BAND II	0.060	0.0300	0.600	1.67
	BAND III	0.072	0.0360	0.720	2.00
TRE	HOMOGENATE	0.030	0.0150	0.300	1.00
	BAND I	0.001	0.0005	0.010	0.33
	BAND II	0.060	0.0300	0.600	2.00
	BAND III	0.065	0.0325	0.650	2.17
FEI	HOMOGENATE	0.017	0.0085	0.170	1.00
	BAND I	0.001	0.0005	0.010	0.06
	BAND II	0.032	0.0160	0.320	1.88
	BAND III	0.030	0.0150	0.300	1.76
ZAI	HOMOGENATE	0.015	0.0075	0.150	1.00
	BAND I	0.001	0.0005	0.010	0.07
	BAND II	0.042	0.0210	0.420	2.80
	BAND III	0.029	0.0145	0.290	1.93
SHU	HOMOGENATE	0.011	0.0055	0.110	1.00
	BAND I	0.009	0.0045	0.090	0.82
	BAND II	0.024	0.0120	0.240	2.18
	BAND III	0.022	0.0110	0.220	2.00

TABLE 8 (b) CONTINUED

ROS	HOMOGENATE	0.027	0.0135	0.270	1.00
	BAND I	0.005	0.0025	0.050	0.19
	BAND II	0.053	0.0265	0.530	1.96
	BAND III	0.040	0.0200	0.400	1.48
ELL	HOMOGENATE	0.018	0.0090	0.180	1.00
	BAND I	0.003	0.0015	0.030	0.17
	BAND II	0.040	0.0200	0.400	2.20
	BAND III	0.032	0.0160	0.320	1.78

*Relative specific activity calculated with respect to homogenate.

TABLE 8 (c)

DISTRIBUTION OF GLUCOSE 6 - PHOSPHATASE ACTIVITY ON SUB-CELLULAR FRACTIONS OBTAINED FROM MICROSOMAL PREPARATION.

PATIENT	FRACTION	O.D.820 nm	uMoles P_i/ 15 mins/ 50 ug PROTEIN	SPECIFIC ACTIVITY uMoles P_i/15mins/ mgPROTEIN	RELATIVE* SPECIFIC ACTIVITY
PER	HOMOGENATE	0.008	0.0040	0.080	1.00
	BAND I	0.013	0.0065	0.130	1.63
	BAND II	0.019	0.0095	0.190	2.36
	BAND III	0.015	0.0075	0.150	1.86

TABLE 8 (c) CONTINUED

VEN	HOMOGENATE	0.014	0.0070	0.140	1.00
	BAND I	0.022	0.0110	0.220	1.57
	BAND II	0.050	0.0250	0.500	3.57
	BAND III	0.030	0.0150	0.300	2.14
MOR	HOMOGENATE	0.009	0.0045	0.090	1.00
	BAND I	0.024	0.0120	0.240	2.67
	BAND II	0.037	0.0185	0.370	4.11
	BAND III	0.024	0.0120	0.240	2.67
OLI	HOMOGENATE	0.013	0.0065	0.130	1.00
	BAND I	0.025	0.0125	0.250	1.92
	BAND II	0.055	0.0285	0.570	4.38
	BAND III	0.038	0.0190	0.380	2.92
TRE	HOMOGENATE	0.015	0.0075	0.150	1.00
	BAND I	0.028	0.0140	0.280	1.86
	BAND II	0.057	0.0295	0.590	3.93
	BAND III	0.035	0.0175	0.350	2.33
FEI	HOMOGENATE	0.010	0.0050	0.100	1.00
	BAND I	0.032	0.0160	0.320	3.20
	BAND II	0.050	0.0250	0.500	5.00
	BAND III	0.041	0.0205	0.410	4.10

TABLE 8 (c) CONTINUED

ZAI	HOMOGENATE	0.012	0.0060	0.120	1.00
	BAND I	0.029	0.0145	0.290	2.42
	BAND II	0.052	0.0260	0.520	4.33
	BAND III	0.045	0.0225	0.450	3.75
SHU	HOMOGENATE	0.015	0.0075	0.150	1.00
	BAND I	0.033	0.0165	0.330	2.20
	BAND II	0.065	0.0325	0.650	4.33
	BAND III	0.049	0.0245	0.490	3.26
ROS	HOMOGENATE	0.019	0.0095	0.190	1.00
	BAND I	0.035	0.0175	0.350	1.84
	BAND II	0.092	0.0460	0.920	4.84
	BAND III	0.081	0.0405	0.810	4.26
ELL	HOMOGENATE	0.018	0.0090	0.180	1.00
	BAND I	0.039	0.0195	0.390	2.16
	BAND II	0.095	0.0475	0.950	5.28
	BAND III	0.076	0.0380	0.760	4.22

*Relative specific activity calculated with respect to homogenate.

TABLE 8 (d)

DISTRIBUTION OF NADH CYTOCHROME C REDUCTASE ACTIVITY ON SUBCELLULAR FRACTIONS OBTAINED FROM MICROSOMAL PREPARATIONS.

PATIENT	TIME SECONDS	O.D. 550 nm			
		HOMOGENATE	BAND I	BAND II	BAND III
PER	0	0.673	0.780	0.905	0.880
	10	0.699	0.820	0.975	1.022
	20	0.727	0.862	1.046	1.160
	30	0.757	0.900	1.125	1.160
	40	0.770	0.935	1.125	1.160
	50	0.783	0.970	1.125	1.160
VEN	0	0.485	0.563	0.670	0.685
	10	0.500	0.597	0.730	0.820
	20	0.523	0.632	0.790	0.945
	30	0.538	0.665	0.855	0.945
	40	0.561	0.695	0.855	0.945
	50	0.588	0.725	0.855	0.945
MOR	0	0.430	0.490	0.590	0.607
	10	0.415	0.525	0.650	0.703
	20	0.465	0.560	0.700	0.818

TABLE 8 (d) CONTINUED

	30	0.481	0.592	0.740	0.823
	40	0.500	0.615	0.745	0.825
	50	0.524	0.643	0.745	0.825
OLI	0	0.462	0.535	0.638	0.635
	10	0.479	0.565	0.795	0.845
	20	0.498	0.605	0.850	0.945
	30	0.513	0.630	0.905	0.950
	40	0.535	0.660	0.905	0.905
	50	0.561	0.690	0.905	0.950
TRE	0	0.445	0.530	0.645	0.650
	10	0.465	0.565	0.715	0.792
	20	0.485	0.610	0.780	0.925
	30	0.508	0.640	0.845	0.925
	40	0.525	0.675	0.845	0.925
	50	0.555	0.705	0.845	0.925
FEI	0	0.425	0.485	0.595	0.610
	10	0.440	0.520	0.650	0.715
	20	0.460	0.555	0.705	0.820
	30	0.480	0.590	0.745	0.825
	40	0.502	0.610	0.750	0.830
	50	0.520	0.635	0.750	0.830

TABLE 8 (d) CONTINUED

ZAI	0	0.505	0.580	0.695	0.710
	10	0.520	0.615	0.760	0.835
	20	0.545	0.665	0.830	0.945
	30	0.565	0.695	0.850	0.945
	40	0.585	0.740	0.850	0.945
	50	0.610	0.760	0.850	0.945
SHU	0	0.360	0.434	0.515	0.553
	10	0.375	0.465	0.564	0.655
	20	0.392	0.500	0.612	0.745
	30	0.414	0.530	0.648	0.751
	40	0.432	0.548	0.652	0.755
	50	0.449	0.570	0.652	0.755
ROS	0	0.440	0.421	0.582	0.688
	10	0.453	0.452	0.644	0.805
	20	0.471	0.480	0.695	0.922
	30	0.492	0.513	0.738	0.925
	40	0.515	0.535	0.740	0.925
	50	0.530	0.552	0.740	0.925
ELL	0	0.405	0.512	0.645	0.668
	10	0.425	0.544	0.702	0.782
	20	0.446	0.580	0.740	0.898
	30	0.461	0.615	0.815	0.945
	40	0.483	0.631	0.815	0.950
	50	0.510	0.675	0.815	0.950

TABLE 8 (e)

SPECIFIC ACTIVITY AND RELATIVE SPECIFIC ACTIVITY OF NADH CYTOCHROME C REDUCTASE.

PATIENT	FRACTION	O.D. 550 nm/ 20 seconds	uMOLES CYT C/min/50 ug PROTEIN	SPECIFIC ACTIVITY uMOLES CYT C/ min/mg PROTEIN	RELATIVE SPECIFIC ACTIVITY
PER	HOMOGENATE	0.058	0.0141	0.141	1.00
	BAND I	0.080	0.0195	0.195	1.38
	BAND II	0.141	0.0343	0.343	2.43
	BAND III	0.280	0.0691	0.691	4.90
VEN	HOMOGENATE	0.050	0.0122	0.244	1.00
	BAND I	0.069	0.0168	0.336	1.38
	BAND II	0.120	0.0292	0.584	2.39
	BAND III	0.261	0.0635	1.270	5.21
MOR	HOMOGENATE	0.043	0.0105	0.210	1.00
	BAND I	0.067	0.0163	0.326	1.55
	BAND II	0.110	0.0268	0.536	2.55
	BAND III	0.211	0.0513	1.026	4.88
OLI	HOMOGENATE	0.048	0.0117	0.234	1.00
	BAND I	0.065	0.0158	0.316	1.35
	BAND II	0.112	0.0272	0.544	2.33
	BAND III	0.210	0.0511	1.022	4.37

TABLE 8 (e) CONTINUED.

TRE	HOMOGENATE	0.047	0.0114	0.228	1.00
	BAND I	0.075	0.0182	0.364	1.60
	BAND II	0.135	0.0328	0.656	2.89
	BAND III	0.275	0.0669	1.338	5.87
FEI	HOMOGENATE	0.040	0.0097	0.194	1.00
	BAND I	0.070	0.0170	0.340	1.75
	BAND II	0.110	0.0268	0.536	2.76
	BAND III	0.210	0.0511	1.022	5.26
ZAI	HOMOGENATE	0.045	0.0110	0.220	1.00
	BAND I	0.085	0.0207	0.414	1.88
	BAND II	0.135	0.0328	0.656	2.98
	BAND III	0.235	0.0572	1.144	5.20
SHU	HOMOGENATE	0.040	0.0097	0.194	1.00
	BAND I	0.066	0.0161	0.322	1.66
	BAND II	0.097	0.0236	0.472	2.43
	BAND III	0.192	0.0467	0.934	4.81
ROS	HOMOGENATE	0.044	0.0107	0.214	1.00
	BAND I	0.061	0.0148	0.296	1.38
	BAND II	0.113	0.0275	0.550	2.57
	BAND III	0.234	0.0569	1.138	5.31

TABLE 8 (e) CONTINUED

ELL	HOMOGENATE	0.049	0.0119	0.238	1.00
	BAND I	0.068	0.0165	0.330	1.39
	BAND II	0.095	0.0231	0.462	1.94
	BAND III	0.230	0.0559	1.118	4.70

TABLE 9 (a)

DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS :

Serum from patient 6 FEI, dated 3 - 2 - 77 used.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.000	29	0.015
2	0.000	30	0.015
3	0.000	31	0.010
4	0.000	32	0.015
5	0.000	33	0.015
6	0.000	34	0.020
7	0.210	35	0.025
8	1.420	36	0.030
9	0.460	37	0.035
10	0.365	38	0.040
11	0.430	39	0.045
12	0.220	40	0.050
13	0.098	41	0.055
14	0.070	42	0.050

TABLE 9 (á) CONTINUED

15	0.050	43	0.045
16	0.040	44	0.040
17	0.030	45	0.035
18	0.025	46	0.035
19	0.020	47	0.035
20	0.020	48	0.030
21	0.020	49	0.030
22	0.025	50	0.030
23	0.025	51	0.025
24	0.025	52	0.020
25	0.025	53	0.020
26	0.025	54	0.015
27	0.020	55	0.010
28	0.020		

* The elution profile is shown graphically in Fig.4 (a). The first peak consisting of fractions 7 - 14 was shown by Ouchterlony double diffusion using anti-human IgG to be IgG fractions.

FIG. 4(a). DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS: Serum from Patient 6 FEI, dated 3-2-77 used.

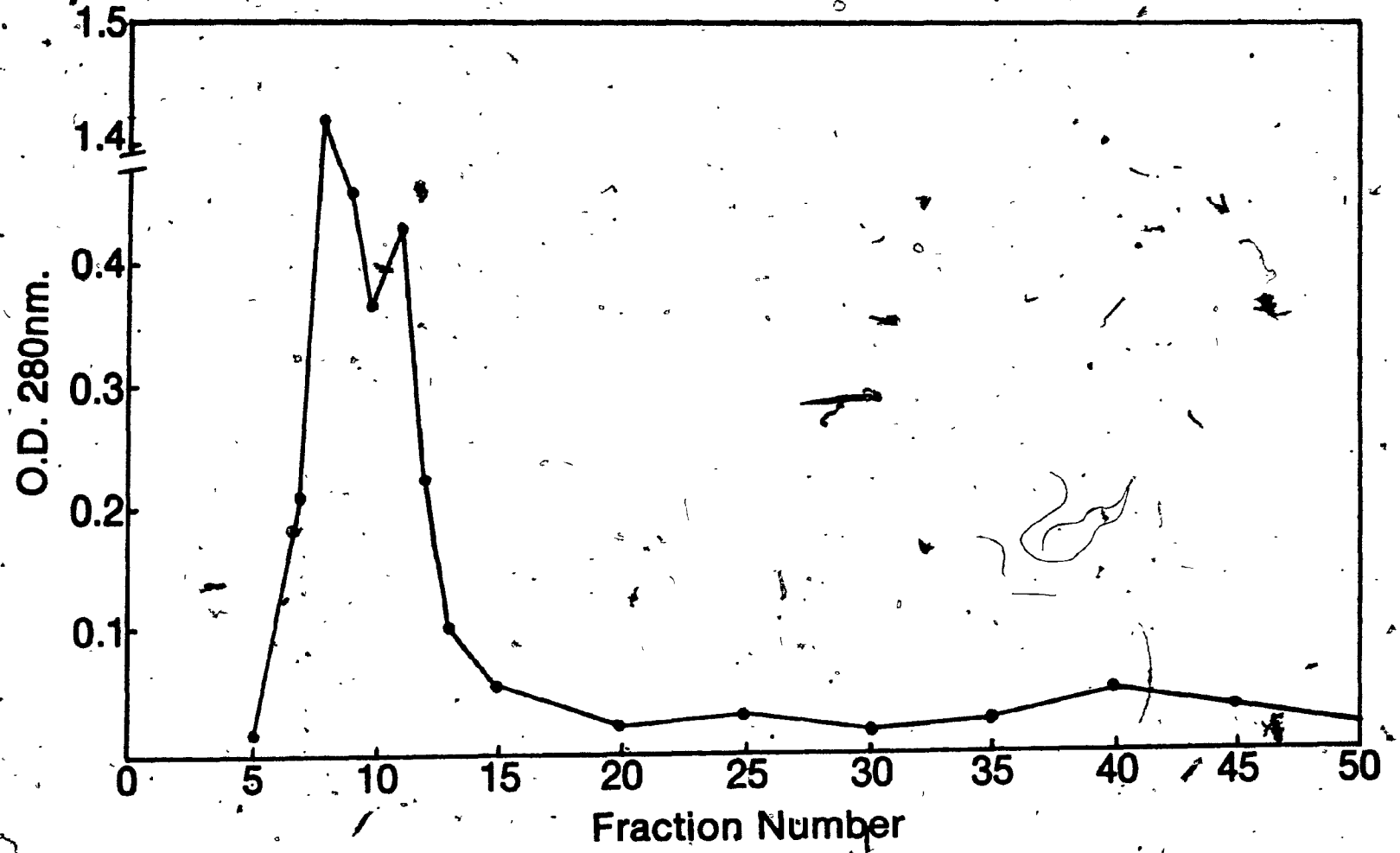


TABLE 9 (b)

DEAE CELLULOSE CHROMATOGRAPY OF IMMUNOGLOBULINS:

Serum from patient 7 ZAI, dated 15 - 2 - 77 used.

FRACTION *	O.D.280 nm	FRACTION	O.D.280 nm
1	0.000	29	0.015
2	0.000	30	0.010
3	0.000	31	0.010
4	0.000	32	0.010
5	0.000	33	0.015
6	0.000	34	0.015
7	0.110	35	0.025
8	1.050	36	0.030
9	0.640	37	0.035
10	0.200	38	0.040
11	0.095	39	0.045
12	0.065	40	0.040
13	0.050	41	0.040
14	0.040	42	0.040
15	0.035	43	0.040
16	0.030	44	0.035
17	0.025	45	0.035
18	0.020	46	0.035
19	0.020	47	0.030
20	0.015	48	0.030

TABLE 9 (b) CONTINUED

21	0.015	49	0.030
22	0.025	50	0.025
23	0.025	51	0.025
24	0.025	52	0.020
25	0.025	53	0.015
26	0.020	54	0.010
27	0.020	55	0.010
28	0.015		

* The elution profile is shown graphically in Fig 4 (b). The first peak consisting of fractions 7 - 13 was shown by Ouchterlony double diffusion using anti - human IgG to be IgG fractions.

FIG. 4(b). DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS: Serum from Patient 7 ZAI, dated 15-2-77 used.

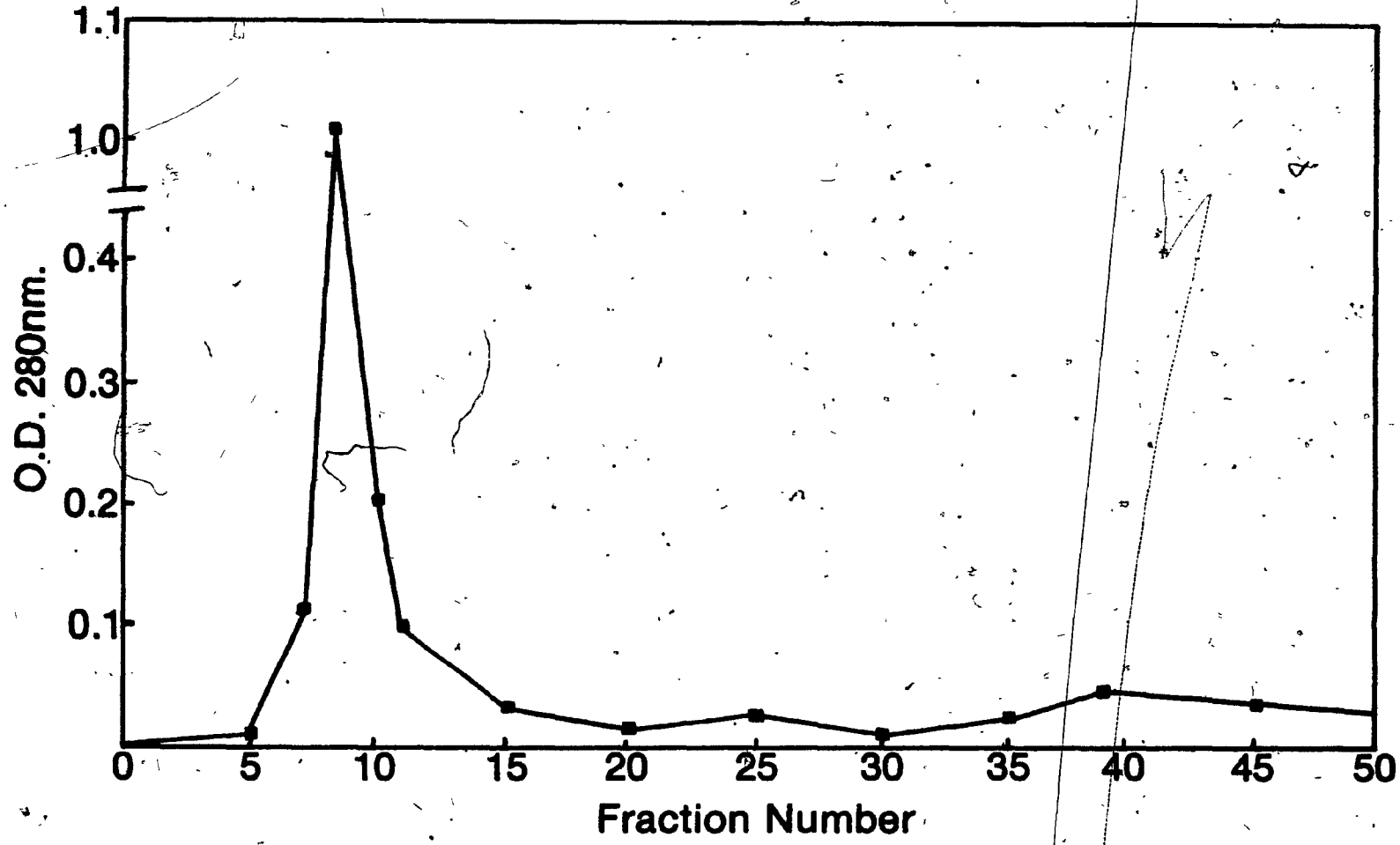


TABLE 9 (c)

DEAE CELLULOSE CHROMATOGRAPY OF IMMUNOGLOBULINS :

Serum from patient 8 SHU, dated 30 - 6 - 76 used.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.000	29	0.020
2	0.000	30	0.025
3	0.000	31	0.015
4	0.000	32	0.010
5	0.000	33	0.010
6	0.240	34	0.015
7	1.540	35	0.020
8	0.380	36	0.025
9	0.360	37	0.030
10	0.230	38	0.040
11	0.165	39	0.045
12	0.055	40	0.050
13	0.025	41	0.060
14	0.020	42	0.070
15	0.020	43	0.055
16	0.020	44	0.045
17	0.015	45	0.035
18	0.015	46	0.035
19	0.015	47	0.035
20	0.015	48	0.030

TABLE 9 (c) CONTINUED

21	0.020	49	0.030
22	0.020	50	0.030
23	0.025	51	0.030
24	0.025	52	0.025
25	0.030	53	0.020
26	0.025	54	0.015
27	0.025	55	0.010
28	0.020		

* The elution profile is shown graphically in Fig 4 (c). The first peak consisting of fractions 6 - 12 was shown by Ouchterlony double diffusion using anti - human IgG to be IgG fractions.

FIG. 4(c). DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS: Serum from Patient 8 SHU, dated 30-6-76 used.

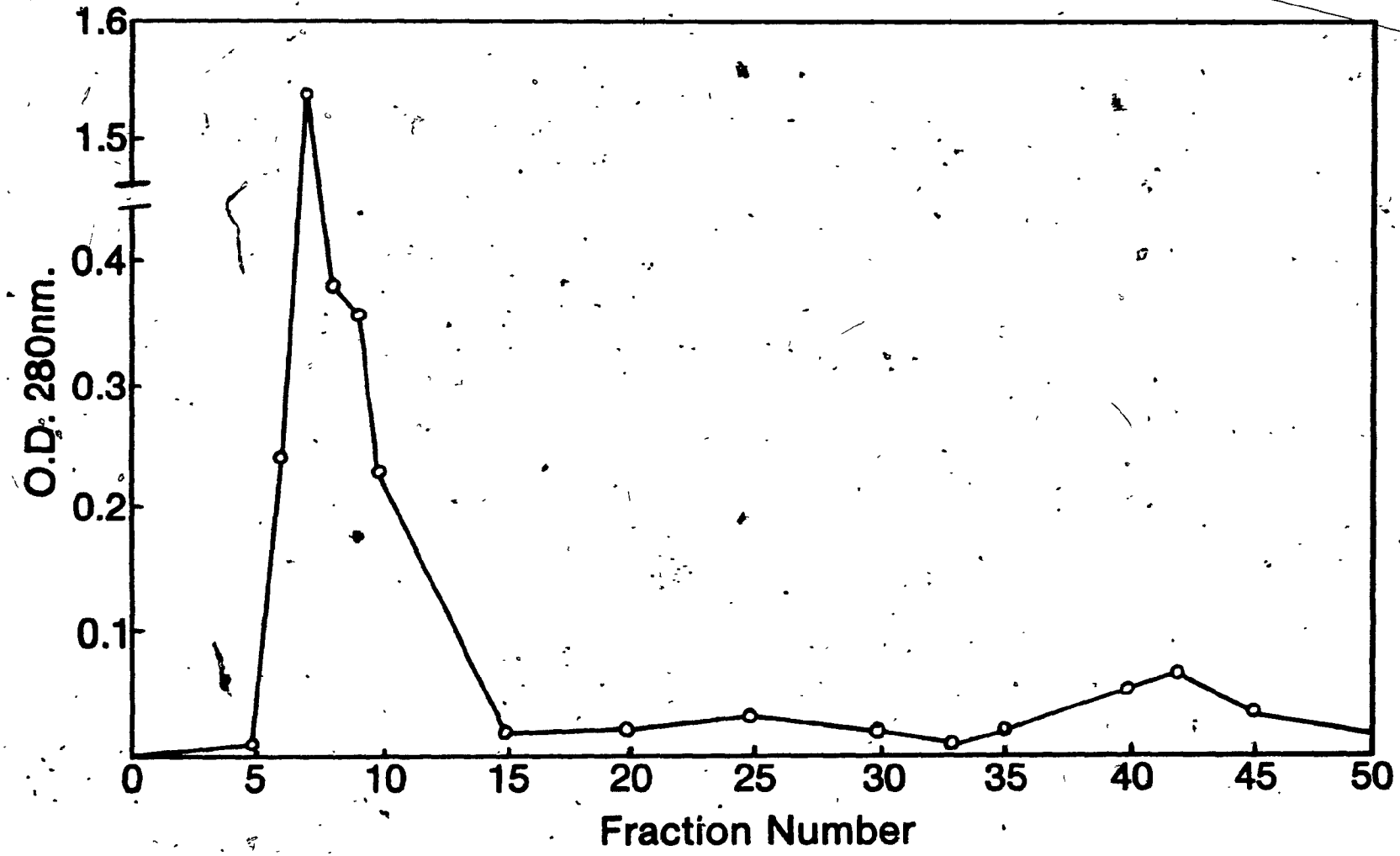


TABLE 9'(d)

DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS:

Serum from patient 9 ROS, dated 3 - 9 - 75 used.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.000	29	0.020
2	0.000	30	0.025
3	0.000	31	0.020
4	0.000	32	0.020
5	0.000	33	0.015
6	0.000	34	0.015
7	0.000	35	0.010
8	1.200	36	0.015
9	0.750	37	0.020
10	0.410	38	0.020
11	0.235	39	0.025
12	0.125	40	0.025
13	0.055	41	0.030
14	0.020	42	0.030
15	0.015	43	0.035
16	0.015	44	0.040
17	0.015	45	0.040
18	0.010	46	0.035
19	0.010	47	0.035
20	0.010	48	0.035

TABLE 9 (d) CONTINUED

21	0.010	49	0.030
22	0.015	50	0.020
23	0.015	51	0.020
24	0.015	52	0.015
25	0.015	53	0.015
26	0.020	54	0.015
27	0.020	55	0.010
28	0.020		

* The elution profile is shown graphically in Fig 4 (d). The first peak consisting of fractions 8 - 13 was shown by Ouchterlony double diffusion using anti - human IgG to be IgG fractions.

FIG. 4(d). DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS: Serum from Patient 9 ROS, dated 3-9-75 used.

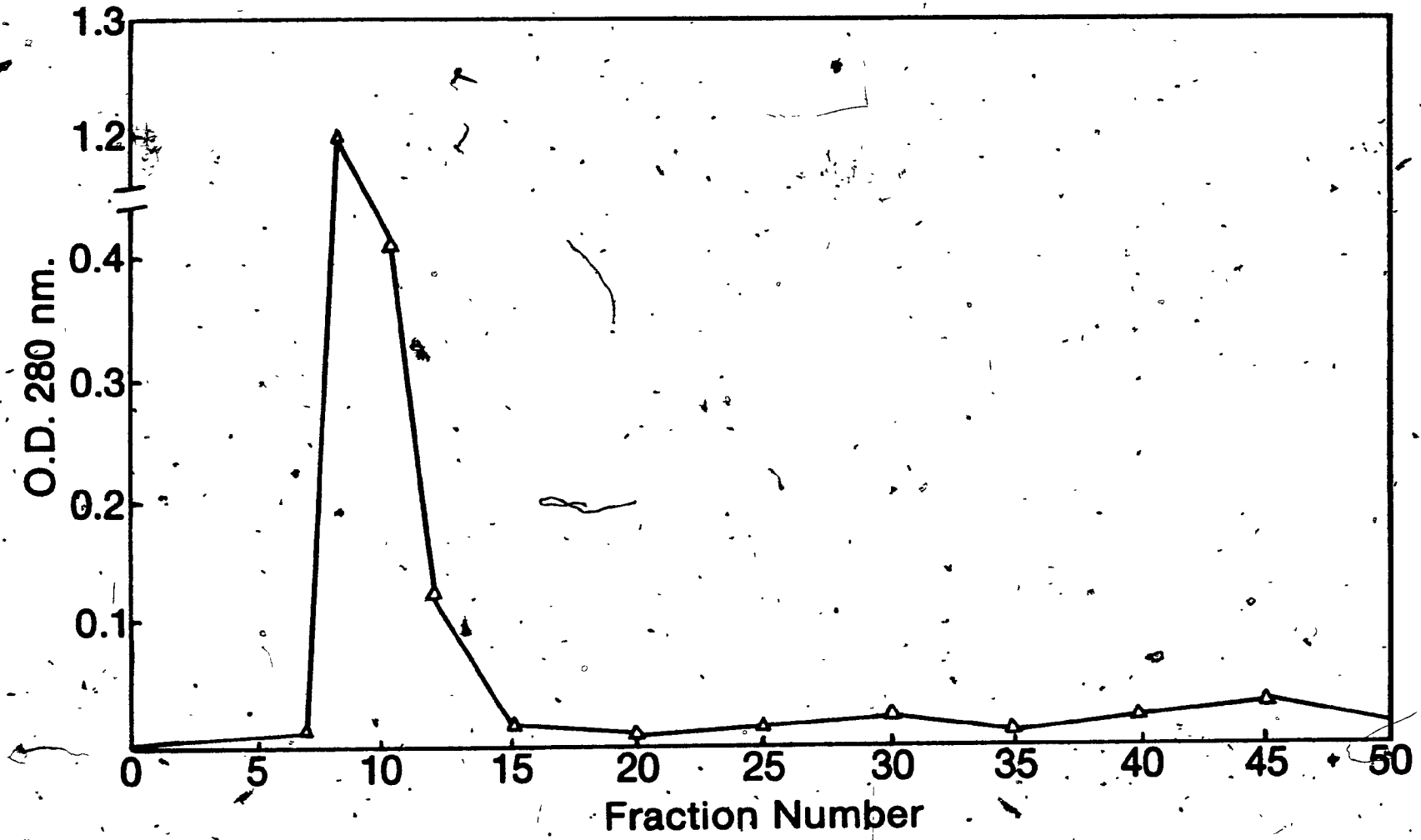


TABLE 9 (e)

DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS:

Serum from patient 10 ELL, dated 10 - 8 - 77 used..

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.000	29	0.030
2	0.000	30	0.030
3	0.000	31	0.025
4	0.000	32	0.020
5	0.000	33	0.015
6	0.000	34	0.020
7	0.150	35	0.020
8	1.190	36	0.025
9	0.650	37	0.025
10	0.440	38	0.030
11	0.160	39	0.040
12	0.080	40	0.055
13	0.050	41	0.065
14	0.040	42	0.050
15	0.040	43	0.045
16	0.040	44	0.040
17	0.040	45	0.035
18	0.035	46	0.035
19	0.035	47	0.030
20	0.030	48	0.030

TABLE 9 (e) CONTINUED

FRACTION	O.D.280 nm	FRACTION	O.D. 280 nm
21	0.025	49	0.025
22	0.025	50	0.025
23	0.025	51	0.020
24	0.020	52	0.020
25	0.020	53	0.010
26	0.020	54	0.010
27	0.025	55	0.010
28	0.032		

* The elution profile is shown graphically in Fig 4 (e). The first peak consisting of fractions 7 - 12 was shown by Ouchterlony double diffusion using anti - human IgG to be IgG fractions.

FIG. 4(e). DEAE CELLULOSE CHROMATOGRAPHY OF IMMUNOGLOBULINS: Serum from Patient 10 ELL, dated 10-8-77 used.

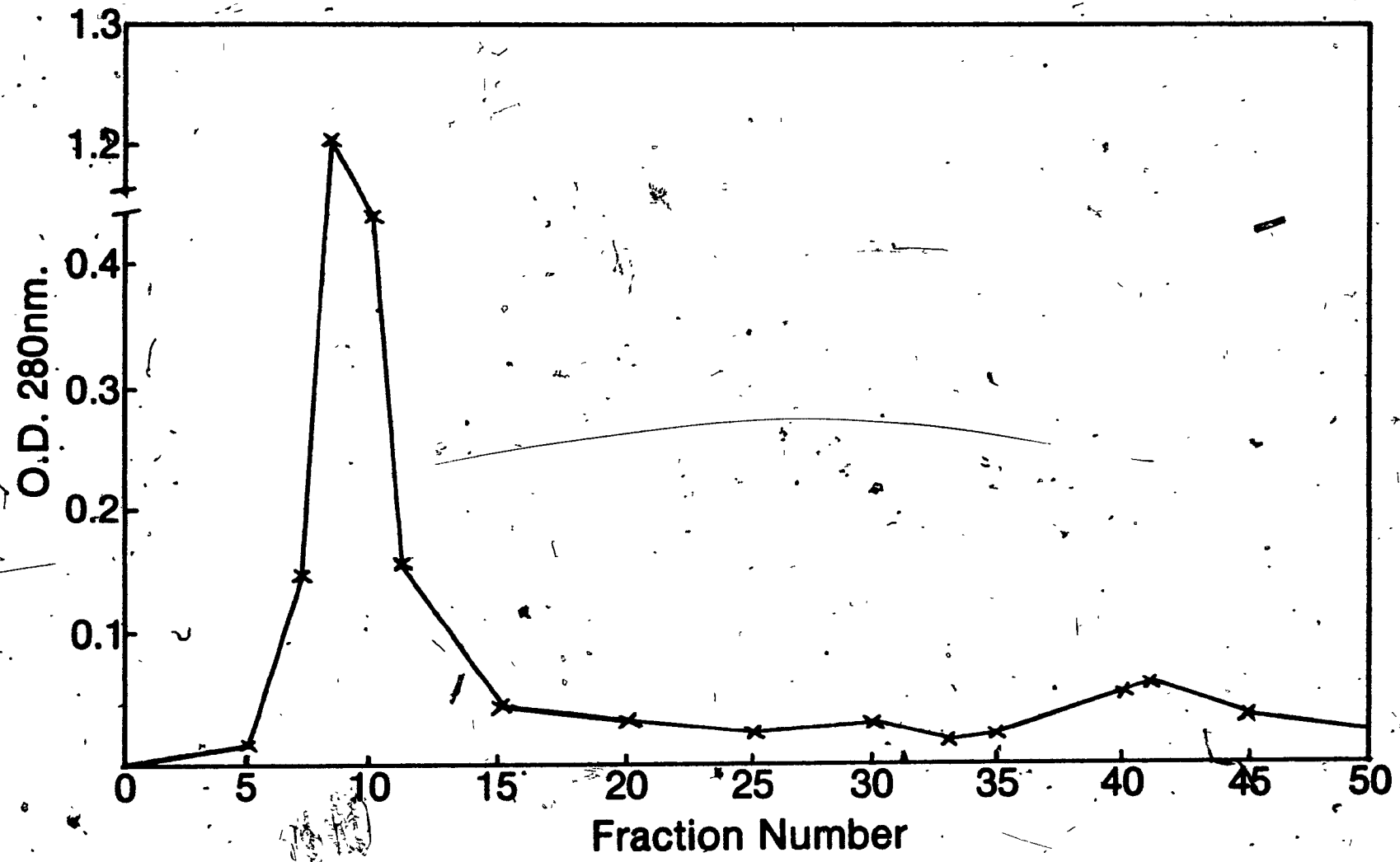


TABLE 9 (f)

DETERMINATION OF IgG FRACTIONS AND CONCENTRATIONS.

PATIENT	IgG FRACTIONS	TOTAL VOLUME	VOLUME AFTER CONCENTRATION	PROTEIN CONCENTRATION
FEI	7 - 14	17.5 ml	1.55 ml	6.6 mg / ml
ZAI	7 - 13	15.1 ml	1.45 ml	6.4 mg / ml
SHU	6 - 12	15.8 ml	1.85 ml	5.5 mg / ml
ROS	8 - 13	14.6 ml	1.75 ml	6.0 mg / ml
ELL	7 - 12	15.5 ml	1.60 ml	6.1 mg / ml

TABLE 10

SOLUBILIZATION OF MICROSOMAL MEMBRANE (BAND A III) FOR AFFINITY CHROMATOGRAPHIC COLUMNS:

Solubilization was accomplished in these cases by treating sonicated membrane at the protein concentration stated with 1% Triton X 100.

PATIENT	INITIAL VOLUME	PROTEIN CONCENTRATION	TOTAL VOLUME AFTER DILUTION	CONCENTRATION AFTER DILUTION
FEI	1 ml	7.4 mg/ml	1.5 ml	4.9 mg/ml
ZAI	1 ml	8.9 mg/ml	1.8 ml	4.9 mg/ml
SHU	1 ml	6.7 mg/ml	1.5 ml	4.4 mg/ml
ROS	1 ml	8.3 mg/ml	1.8 ml	4.6 mg/ml

TABLE 10 CONTINUED

ELL	1 ml	8.1 mg/ml	1.8 ml	4.4 mg/ml
FEI	1 ml	7.7 mg/ml	1.8 ml	4.2 mg/ml

Microsomal membrane from each patient was tested against autologous serum by countercurrent immunoelectrophoresis and Ouchterlony before and after solubilization. Positive immunoprecipitation was observed in all cases. (See Plates 1 and 2)

TABLE 11 (a)

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 6 FEI (FEI I).

FRACTION	O.D. 280 nm
1	0.100
2	0.135
3	0.045
4	0.000
5	0.000
6	0.000
7	0.000
8	0.000
9	0.000
10	0.000
ADDITION OF SOLUBILIZED ANTIGEN FLOW OCCLUDED FOR 1 HOUR	
28	0.125
29	0.090
30	0.025
31	0.000
32	0.000
POTASSIUM THIOCYANATE (KSCN) ADDED ABS.KSCN = 0.055	
33	0.000
34	0.000
35	0.000
36	0.032

TABLE 11 (a) CONTINUED

11	0.000	37	0.045
12	0.000	38	0.055
13	0.025	39	0.060
14	0.105	40	0.060
15	0.180	41	0.055
16	0.185	42	0.055
17	0.185	43	0.055
18	0.190	44	0.055
19	0.195	45	0.055
20	0.185	46	0.055
21	0.175	47	0.055
22	0.175	48	0.055
23	0.170	49	0.055
24	0.165	50	0.055
25	0.160	51	0.055
26	0.155	52	0.055
27	0.150	53	0.055
		54	0.055

* The elution profile is shown graphically in Fig.5 (a). The inset depicts the antigenic fractions after each fraction was dialyzed to remove the KSCN. Fractions 36 - 40 were shown by immunoelectrophoresis to be antigenic.

FIG. 5(a). ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 6 FEI, (FEI I).

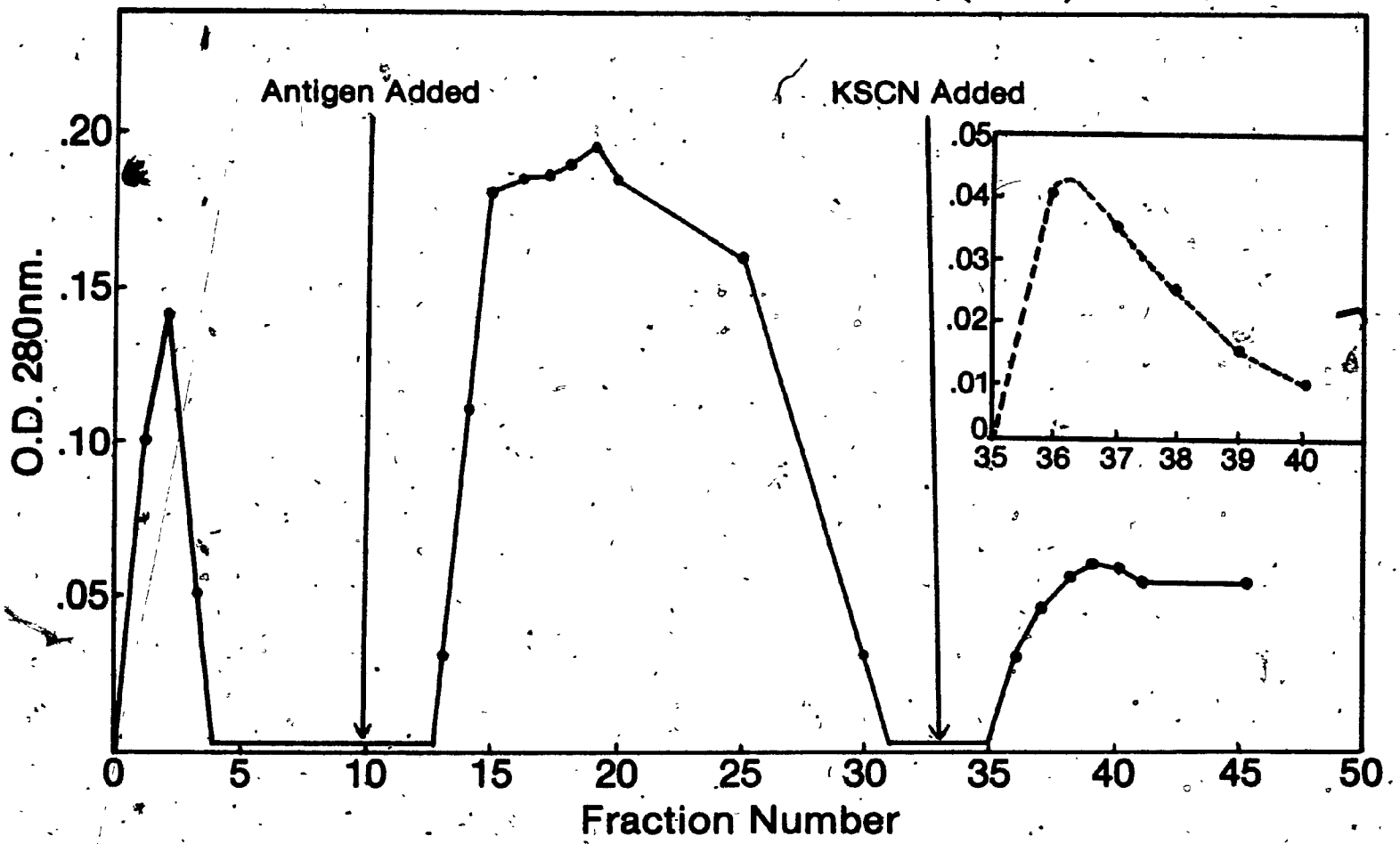


TABLE 11 (b)

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 6 FEI. USING SERA FROM PATIENT ELL AS THE SOURCE OF ANTIBODY (FEI II).

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.095	26	0.000
2	0.120	27	0.000
3	0.035	28	0.000
4	0.000	29	0.000
5	0.000	30	0.000
6	0.000		
7	0.000	POTASSIUM THIOCYNATE (KSCN)	
8	0.000	ADDED ABS. KSCN=0.060	
9	0.000		
ADDITION OF SOLUBILIZED ANTIGEN		31	0.000
		32	0.000
FLOW OCCLUDED FOR 1 HOUR		33	0.000
10	0.000	34	0.015
11	0.000	35	0.045
12	0.245	36	0.060
13	0.295	37	0.060
14	0.135	38	0.060
15	0.095	39	0.060
16	0.085	40	0.060

TABLE 11 (b) CONTINUED

17	0.070	41	0.060
18	0.060	42	0.060
19	0.050	43	0.060
20	0.045	44	0.060
21	0.030	45	0.060
22	0.025	46	0.060
23	0.020	47	0.060
24	0.010	48	0.060
25	0.000	49	0.060
		50	0.060
		51	0.060
		52	0.060

* The elution profile is shown graphically in Fig.5(b). The inset depicts the antigenic fractions after each fraction was dialyzed to remove the KSCN. Fractions 34 - 36 were shown by immunoelectrophoresis to be antigenic.

FIG. 5(b). ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 6 FEI. (FEI II).

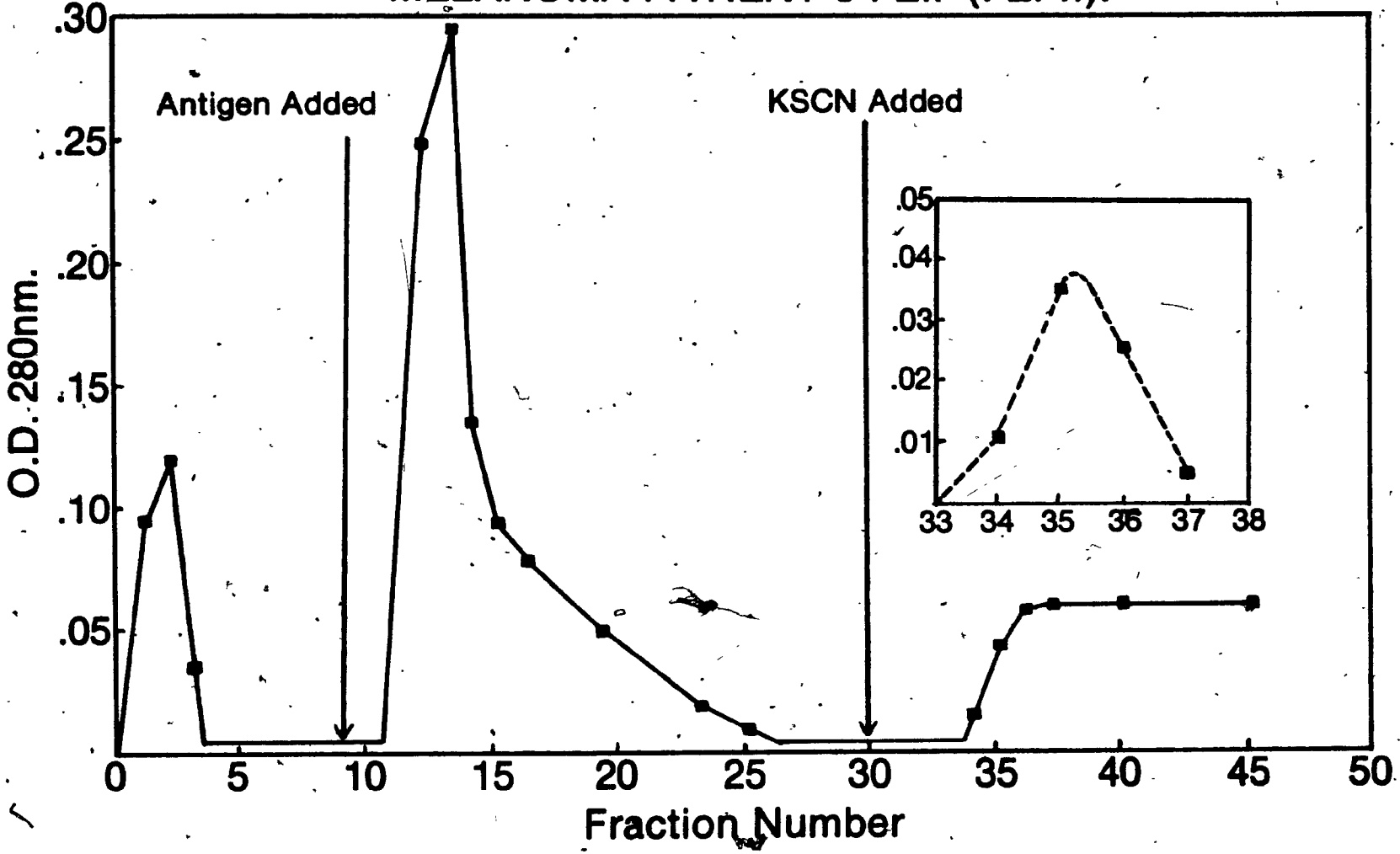


TABLE 12

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 7 ZAI.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.090	29	0.000
2	0.005	30	0.000
3	0.000	31	0.000
4	0.000		
5	0.000	POTASSIUM THIOCYNATE (KSCN)	
6	0.000	ADDED ABS. KSCN=0,075	
7	0.000		
8	0.000		
9	0.000	32	0.000
		33	0.000
ADDITION OF SOLUBILIZED ANTIGEN		34	0.000
FLOW OCCLUDED FOR 1 HOUR		35	0.000
		36	0.000
10	0.000	37	0.000
11	0.185	38	0.000
12	0.200	39	0.000
13	0.185	40	0.025
14	0.185	41	0.055
15	0.165	42	0.065
16	0.135	43	0.075

TABLE 12 CONTINUED

17	0.100	44	0.080
18	0.050	45	0.085
19	0.025	46	0.075
20	0.050	47	0.075
21	0.010	48	0.075
22	0.010	49	0.075
23	0.010	50	0.075
24	0.025	51	0.075
25	0.000	52	0.075
26	0.000	53	0.075
27	0.000	54	0.075
28	0.000	55	0.075

* The elution profile is shown graphically in Fig. 6. The inset depicts the antigenic fractions after each fraction was dialyzed to remove the KSCN. Fractions 42, 44 and 45 were shown by immunoelectrophoresis to be antigenic.

FIG. 6 ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA. PATIENT 7 ZAI.

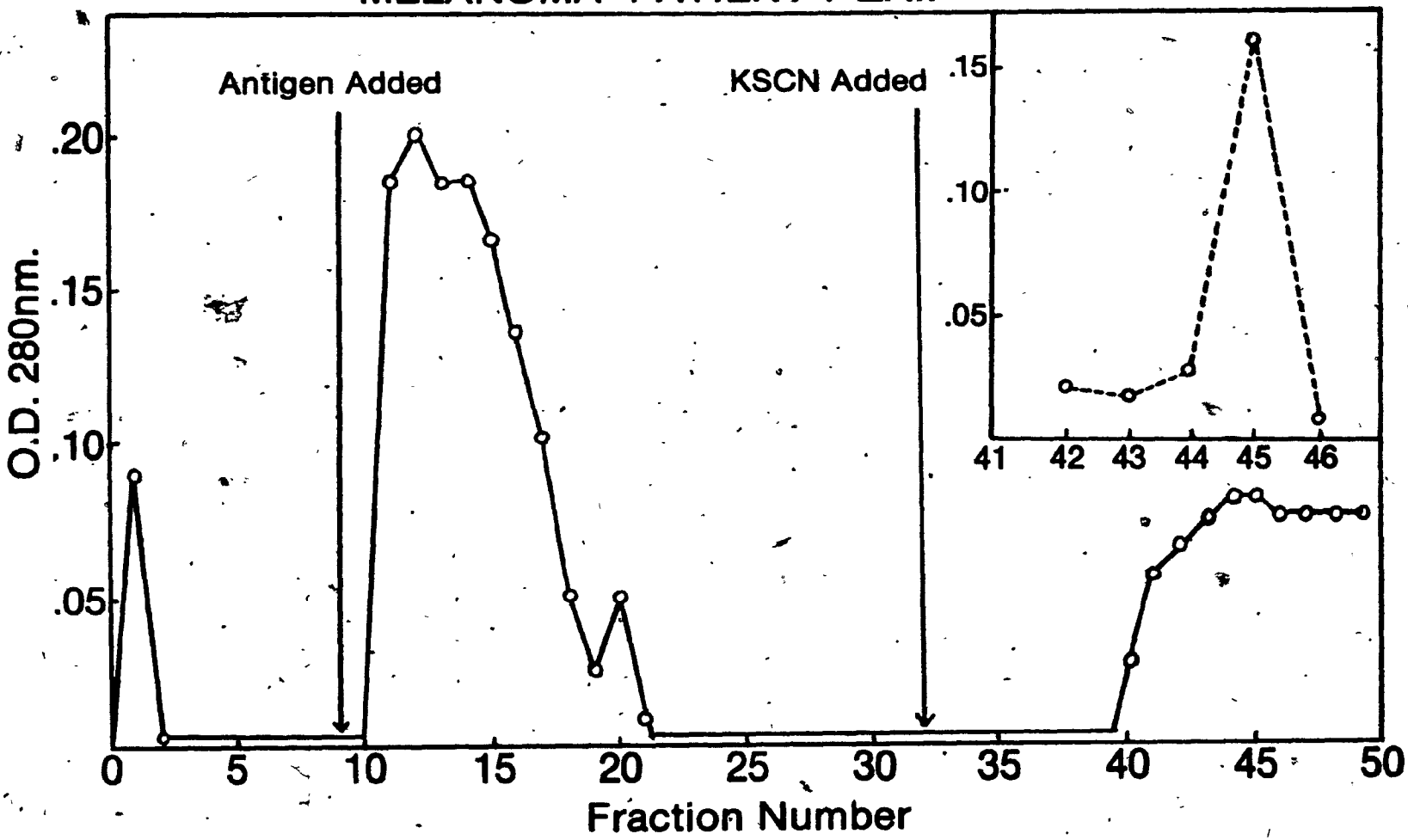


TABLE 13

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT

8 SHU.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.130	29	0.000
2	0.055	30	0.000
3	0.040		
4	0.020	POTASSIUM THIOCYNATE (KSCN)	
5	0.005		
6	0.000	ADDED ABS. KSCN=0.055	
7	0.000		
8	0.000	31	0.000
9	0.000	32	0.000
10	0.000	33	0.000
		34	0.000
ADDITION OF SOLUBILIZED ANTIGEN		35	0.000
		36	0.035
FLOW OCCLUDED FOR 1 HOUR		37	0.075
		38	0.070
11	0.000	39	0.070
12	0.065	40	0.065
13	0.260	41	0.055
14	0.240	42	0.055
15	0.175	43	0.055

TABLE 13 CONTINUED

16	0.175	44	0.055
17	0.175	45	0.055
18	0.140	46	0.055
19	0.145	47	0.055
20	0.125	48	0.055
21	0.090	49	0.055
22	0.055	50	0.055
23	0.020	51	0.055
24	0.000	52	0.055
25	0.000	53	0.055
26	0.000	54	0.055
27	0.000	55	0.055
28	0.000		

* The elution profile is shown graphically in Fig. 7. The inset depicts the antigenic fractions after each fraction was dialyzed to remove the KSCN. Fractions 36 - 40 were shown by immunoelectrophoresis to be antigenic.

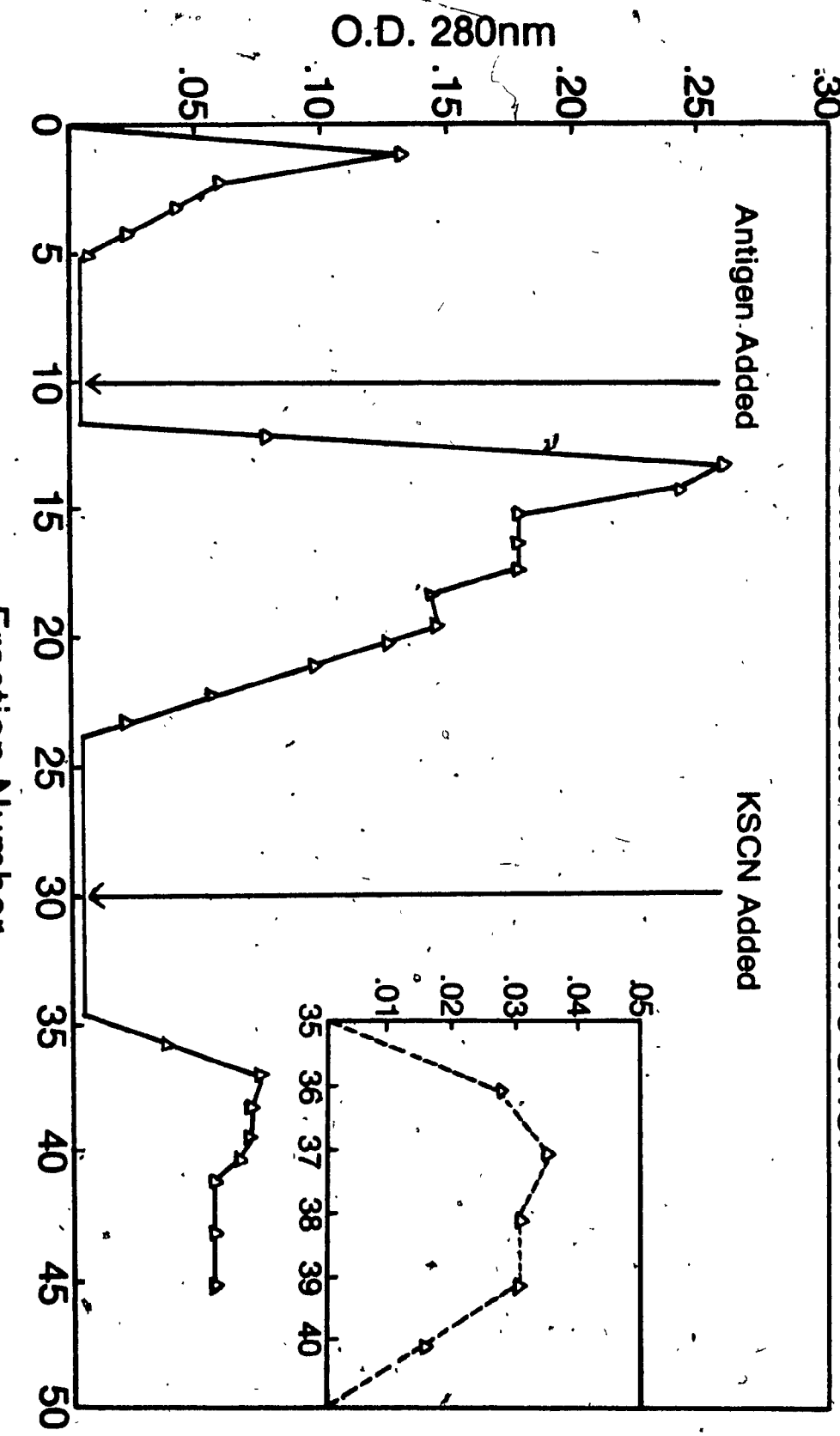


FIG.7 ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 8 SHU.

TABLE 14

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT

9 ROS.

FRACTION *	O.D. 280 nm	FRACTION	O.D. 280 nm
1	0.096	POTASSIUM THIOCYANATE (KSCN)	
2	0.035		
3	0.000	ADDED ABS. KSCN=0.065	
4	0.000		
5	0.000	29	0.000
6	0.000	30	0.000
7	0.000	31	0.000
8	0.000	32	0.000
9	0.000	33	0.000
10	0.000	34	0.024
ADDITION OF SOLUBILIZED ANTIGEN		35	0.065
		36	0.085
FLOW OCCLUDED FOR 1 HOUR		37	0.075
		38	0.070
		39	0.065
11	0.000	40	0.065
12	0.150	41	0.065
13	0.290	42	0.065
14	0.285	43	0.065
15	0.168	44	0.065

TABLE 14 CONTINUED

16	0.066	45	0.065
17	0.062	46	0.065
18	0.040	47	0.065
19	0.025	48	0.065
20	0.016	49	0.065
21	0.000	50	0.065
22	0.000	51	0.065
23	0.000	52	0.065
24	0.000	53	0.065
25	0.000	54	0.065
26	0.000	55	0.065
27	0.000		
28	0.000		

* The elution profile is shown graphically in Fig. 8. The inset depicts the antigenic fractions after each was dialyzed to remove the KSCN. Fractions 34 - 38 were shown by immunoelectrophoresis to be antigenic.

FIG. 8 ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 9 ROS.

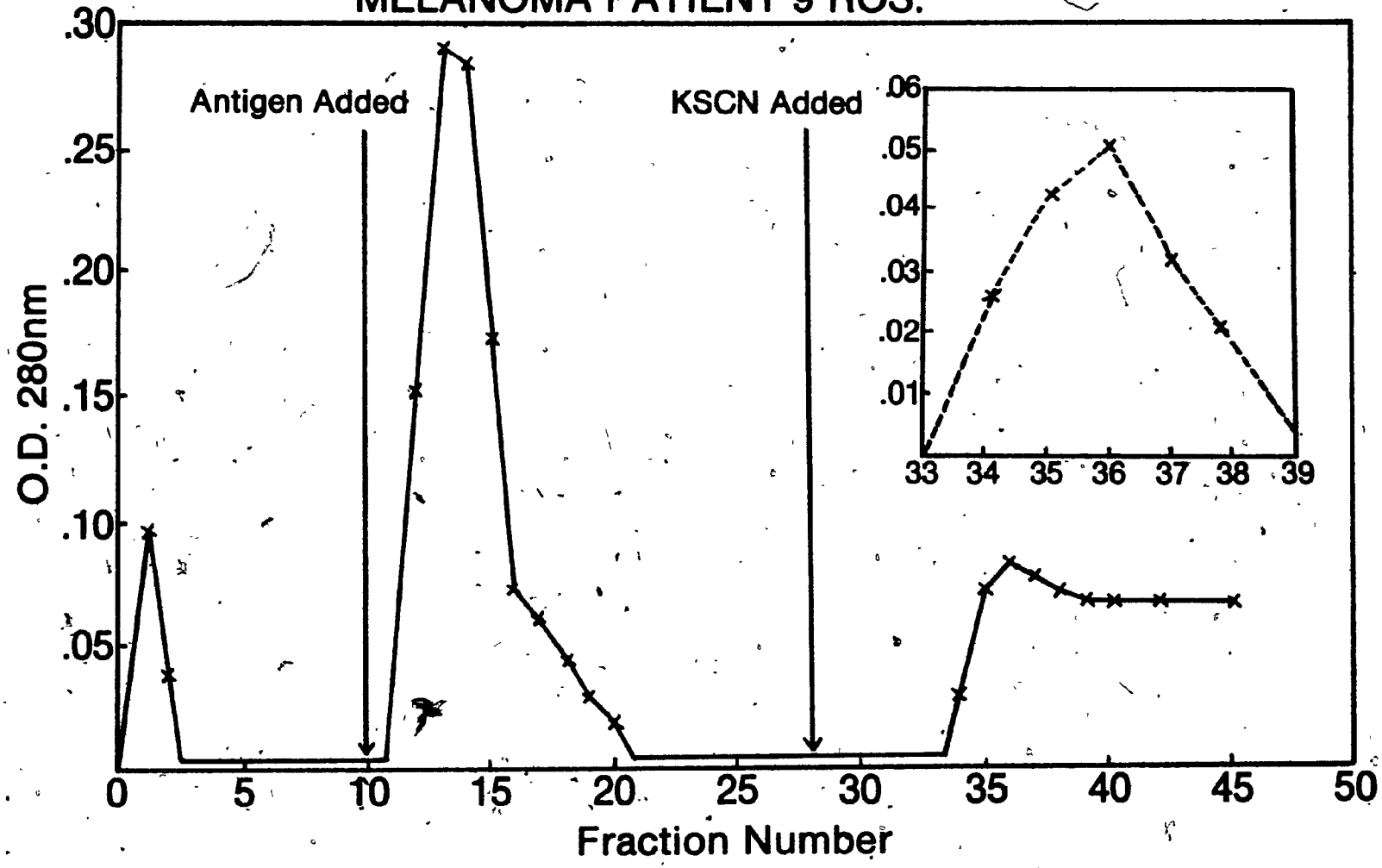


TABLE 15

ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT

10 ELL.

FRACTION *	O.D. 280 nm
1	0.085
2	0.025
3	0.015
4	0.015
5	0.000
6	0.000
7	0.000
8	0.000
9	0.000
10	0.000
ADDITION OF SOLUBILIZED ANTIGEN	
FLOW OCCLUDED FOR 1 HOUR	
11	0.000
12	0.000
13	0.455
14	0.475
15	0.410

FRACTION	O.D. 280 nm
29	0.000
30	0.000
31	0.000
32	0.000
33	0.000
POTASSIUM THIOCYANATE (KSCN)	
ADDED ABS. KSCN=0.060	
34	0.000
35	0.000
36	0.000
37	0.000
38	0.043
39	0.065
40	0.070
41	0.060
42	0.060
43	0.060

TABLE 15 CONTINUED

16	0.400	44	0.060
17	0.370	45	0.060
18	0.375	46	0.060
19	0.350	47	0.060
20	0.280	48	0.060
21	0.288	49	0.060
22	0.178	50	0.060
23	0.100	51	0.060
24	0.054	52	0.060
25	0.022	53	0.060
26	0.005	54	0.060
27	0.000	55	0.060
28	0.000		

*. The elution profile is shown graphically in Fig. 9. The inset depicts the antigenic fractions after each was dialyzed to remove the KSCN. Fractions 38 - 40 were shown by immunoelectrophoresis to be antigenic.

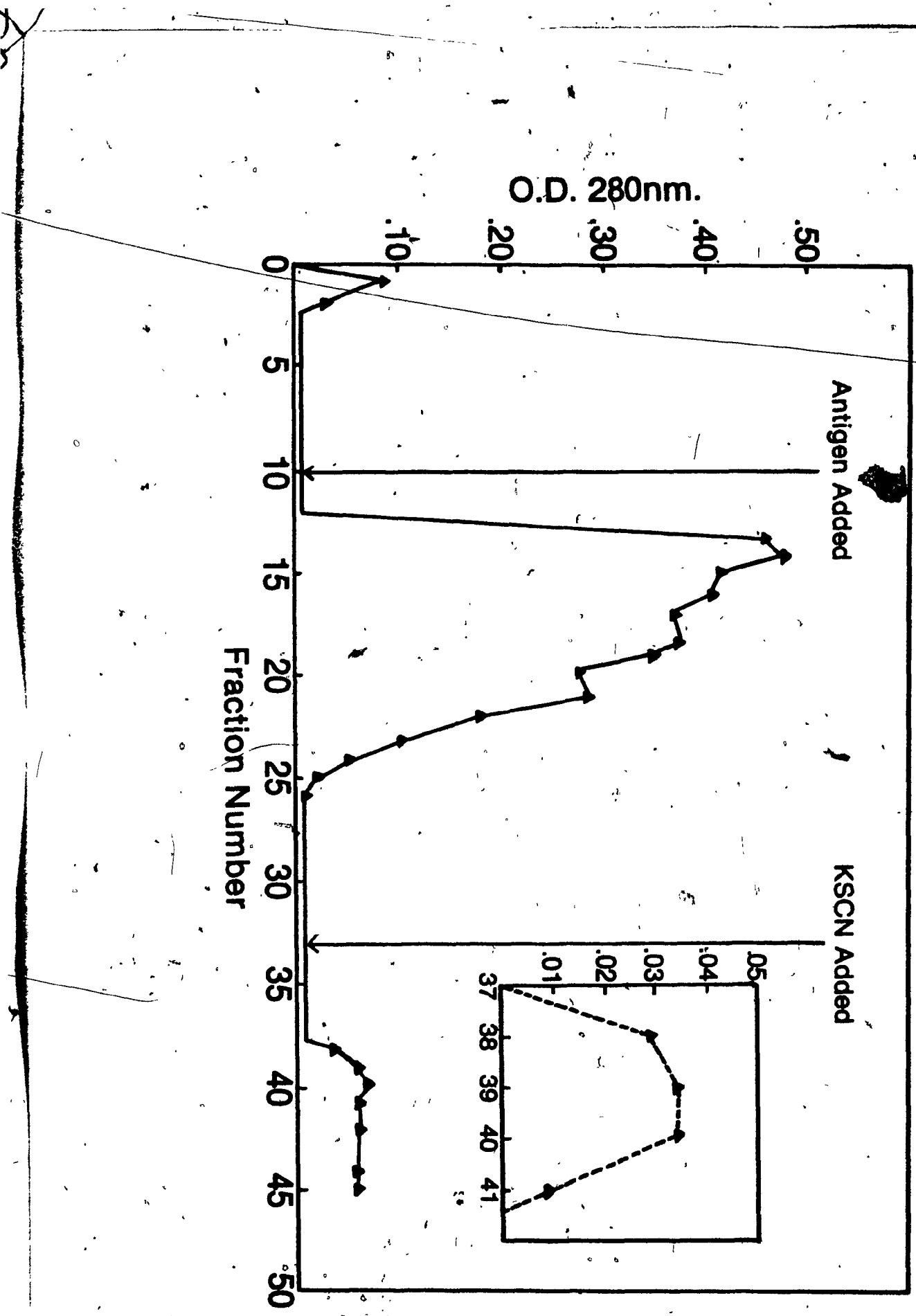


FIG. 9 ISOLATION OF MICROSOMAL ANTIGEN FROM MELANOMA PATIENT 10 ELL.

TABLE 16

PROTEIN CONCENTRATION OF ANTIGENIC FRACTIONS:

Each fraction was dialyzed against 0.01 M potassium phosphate, 0.15 M NaCl pH 7.5 for 12 - 14 hours in a cold room at 4° C. Protein concentration of each fraction was determined by the Lowry method.

PATIENT	FRACTION	O.D. 600 nm	POOLED VOLUME ml	VOLUME AFTER CONCENTRATION ml	PROTEIN CONCENTRATION IN CONCENTRATED FRACTION O.D.600 nm	mg / ml
FEI I	36	0.041/50ul	36-40			
	37	0.035/50ul				
	38	0.025/50ul	4.5	0.75	0.065/50ul	0.26
	39	0.015/50ul				
	40	0.010/50ul				
ZAI	40	0.015/50ul				
	41	0.015/50ul	42,44,45			
	42	0.020/50ul				
	43	0.015/50ul	2.5	0.60	0.110/50ul	0.47
	44	0.025/50ul				
	45	0.155/50ul				
SHU	36	0.028/50ul	36-40			
	37	0.035/50ul				

TABLE 16 CONTINUED

	38	0.030/50ul	4.3	0.65	0.058/50ul	0.24
	39	0.030/50ul				
	40	0.015/50ul				
ROS	34	0.025/50ul	34-38			
	35	0.042/50ul				
	36	0.051/50ul	4.7	0.56	0.055/50ul	0.23
	37	0.035/50ul				
	38	0.017/50ul				
ELL	38	0.030/50ul	38-40			
	39	0.035/50ul				
	40	0.035/50ul	2.5	0.48	0.049/50ul	0.22
	41	0.010/50ul				
FEI II	34	0.011/50ul	34-36			
	35	0.035/50ul				
	36	0.025/50ul	2.8	0.35	0.053/50ul	0.22
	37	0.005/50ul				

TABLE 17

IMMUNOLOGICAL TEST (IMMUNOELECTROPHORESIS) ON ANTIGENS WITH AUTOLOGOUS, ALLOGENEIC MELANOMA AND CONTROL SERA. Before testing, a 200 ul aliquot of each antigen fraction was removed and further concentrated to at least one - half of this volume.

PATIENT	ANTIGEN DILUTION	SERA USED				
		FEI	ZAI	SHU	SARCOMA	NORMAL
FEI I	1/2	3+	1+	2+	-	-
	1/4	3+	-	2+	-	-
ZAI	1/2	1+	3+	1+	-	-
	1/4	-	3+	-	-	-
SHU	1/2	2+	2+	3+	-	-
	1/4	2+	2+	3+	-	-
		ROS	ELL	FEI		
ROS	1/2	3+	2+	1+	-	-
	1/4	3+	2+	-	-	-
ELL	1/2	2+	3+	1+	-	-
	1/4	2+	3+	-	-	-
FEI II	1/2	3+	2+	3+	-	-
	1/4	3+	2+	3+	-	-

LEGEND: 3+ strong, 2+ weak and 1+ very weak immuno precipitation line. - no immunoprecipitation lines observed.

TABLE 18

SEPARATION OF ANTIGEN - ANTIBODY COMPLEXES ON SEPHADEX G 200 COLUMN:

23.5 mg of 50% ammonium sulfate precipitated protein from negative sera of patient 6 FEI were applied to the column. The column was eluted with 0.2 M Glycine HCl pH 2.2 and 2 ml fractions collected. Each fraction was subsequently dialyzed against PBS pH 7.5 and protein concentration done on each one. All fractions were tested, using double diffusion Ouchterlony against autologous positive sera and anti-human IgG.

FRACTION	O.D. 600 nm	IMMUNOLOGICAL PATTERN	
		ANTI - HUMAN IgG	FEI SERA
1	0.020/20ul	-	-
2	0.020	-	-
3	0.020	-	-
4	0.020	-	-
5	0.035	-	-
6	0.195	-	-
7	0.640	-	-
8	0.850	-	-
9	1.250	-	-
10	0.860	-	-

TABLE 18 CONTINUED

11	0.800	+	-
12	0.680	+	-
13	0.660	+	-
14	0.640	+	-
15	0.630	+	-
16	0.480	+	-
17	0.350	+	-
18	0.175	+	-
19	0.085	+	-
20	0.020	+	-
21	0.015	-	+
22	0.020	-	+
23	0.035	-	+
24	0.105	-	+
25	0.078	-	+
26	0.020	-	+
27	0.015	-	-

PLATE 1

(a) Countercurrent Immunoelectrophoresis on Autologous and Allogeneic antigens isolated from 1% Triton X 100 solubilized membrane from Patient FEI. Wells 1 - 6 contain antigens from fractions 35 - 40 obtained from FEI I.* Wells 7 - 11 contain antigens from fractions 33 - 37 obtained from FEI II.** Wells 12 contain antigen from FEI Immune complexes. All antigen fraction tested against Autologous FEI serum.

*Table IIa **Table IIb

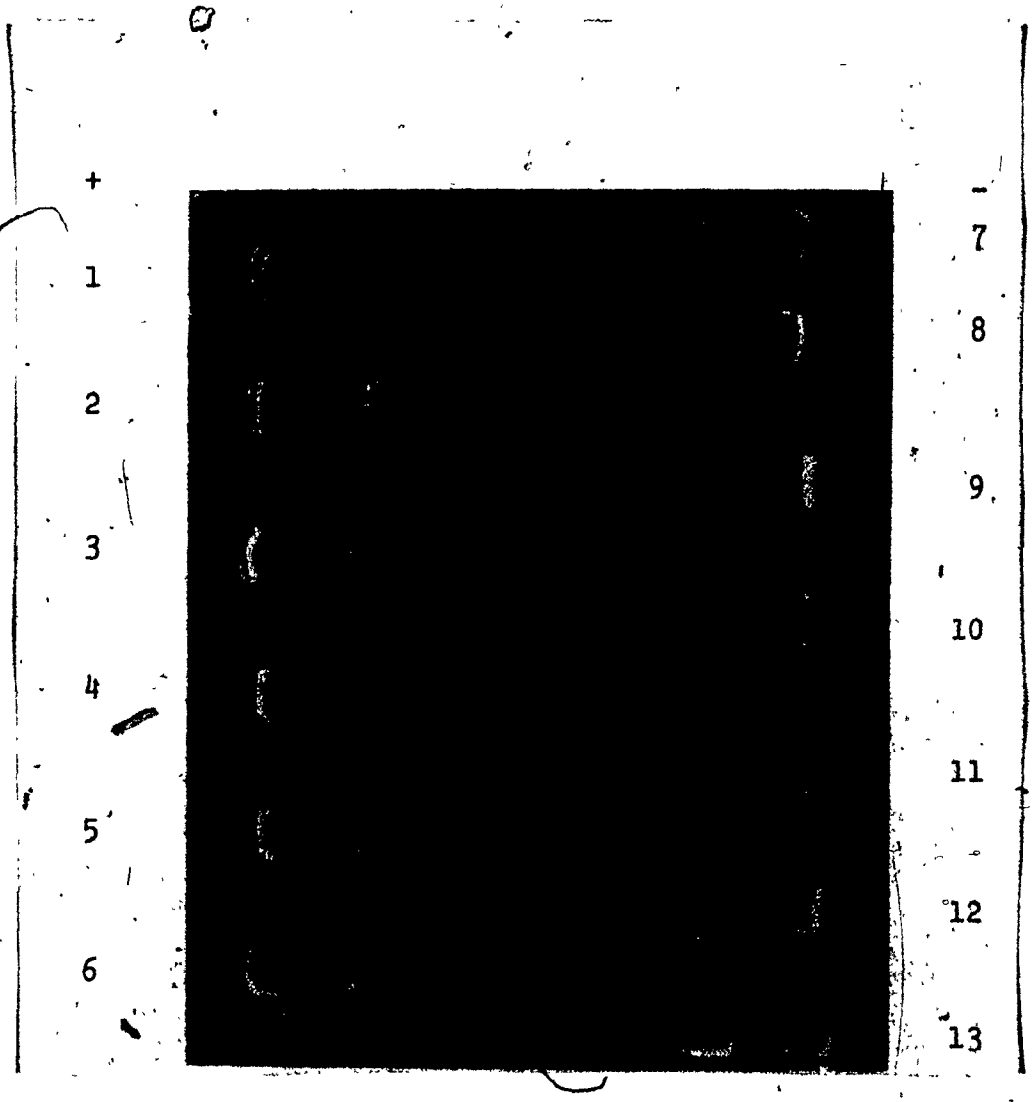
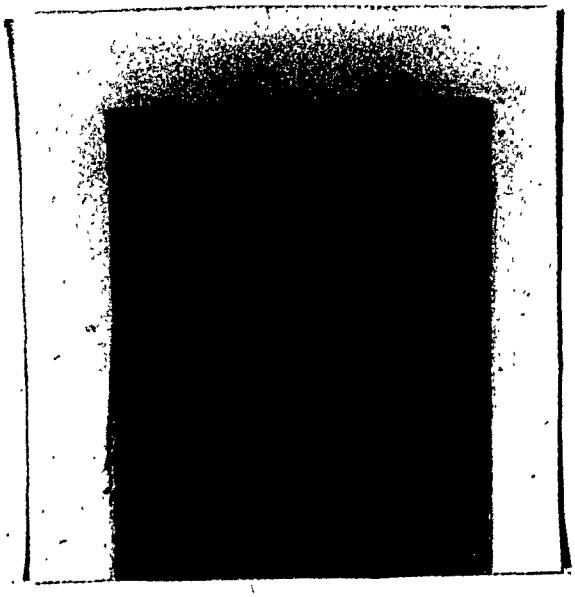


Plate 1

(a)



(b)

PLATE 2

(a) Ouchterlony (Double-diffusion precipitation) test on 1% Triton X 100 solubilized microsomal membrane from patient FEI. Centre well contains solubilized membrane. Wells 1 and 3 contain serum from a teratoma and a normal patient respectively. Wells 2 and 4 contain serum from melanoma patient FEI and ROS respectively.

(b) Ouchterlony plate showing IgG positive fractions obtained from DEAE cellulose chromatography of melanoma patient FEI serum. Fractions # 5 to 10.

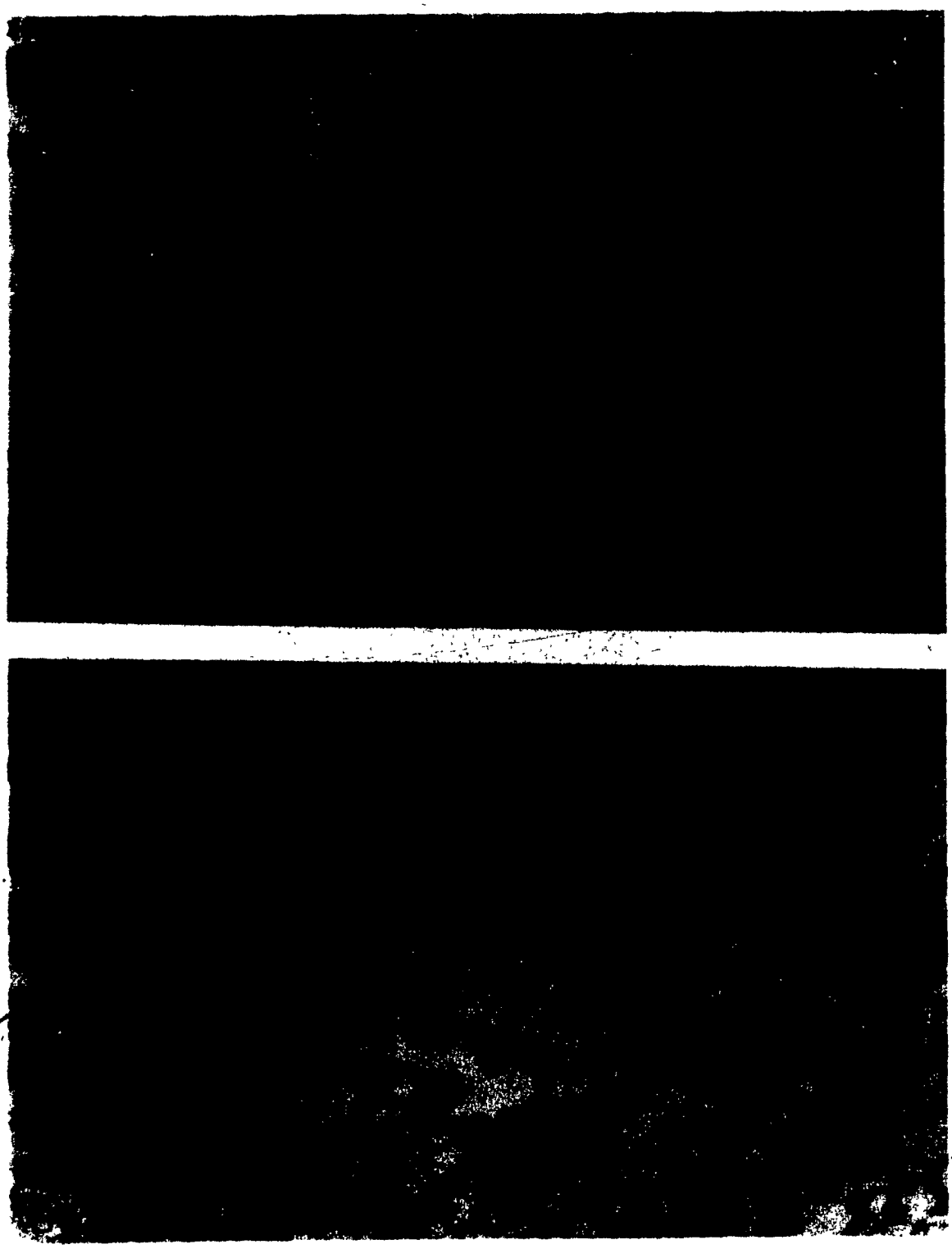


PLATE 3

Electron Micrographs of rough endoplasmic reticulum isolated from melanoma cells. Pellets were resuspended in 0.25 M Sucrose and processed for electron microscopy. X 39,000.
a) FEI b) ELL

FIG. 10 CALIBRATION CURVE FOR 5.6% GELS.

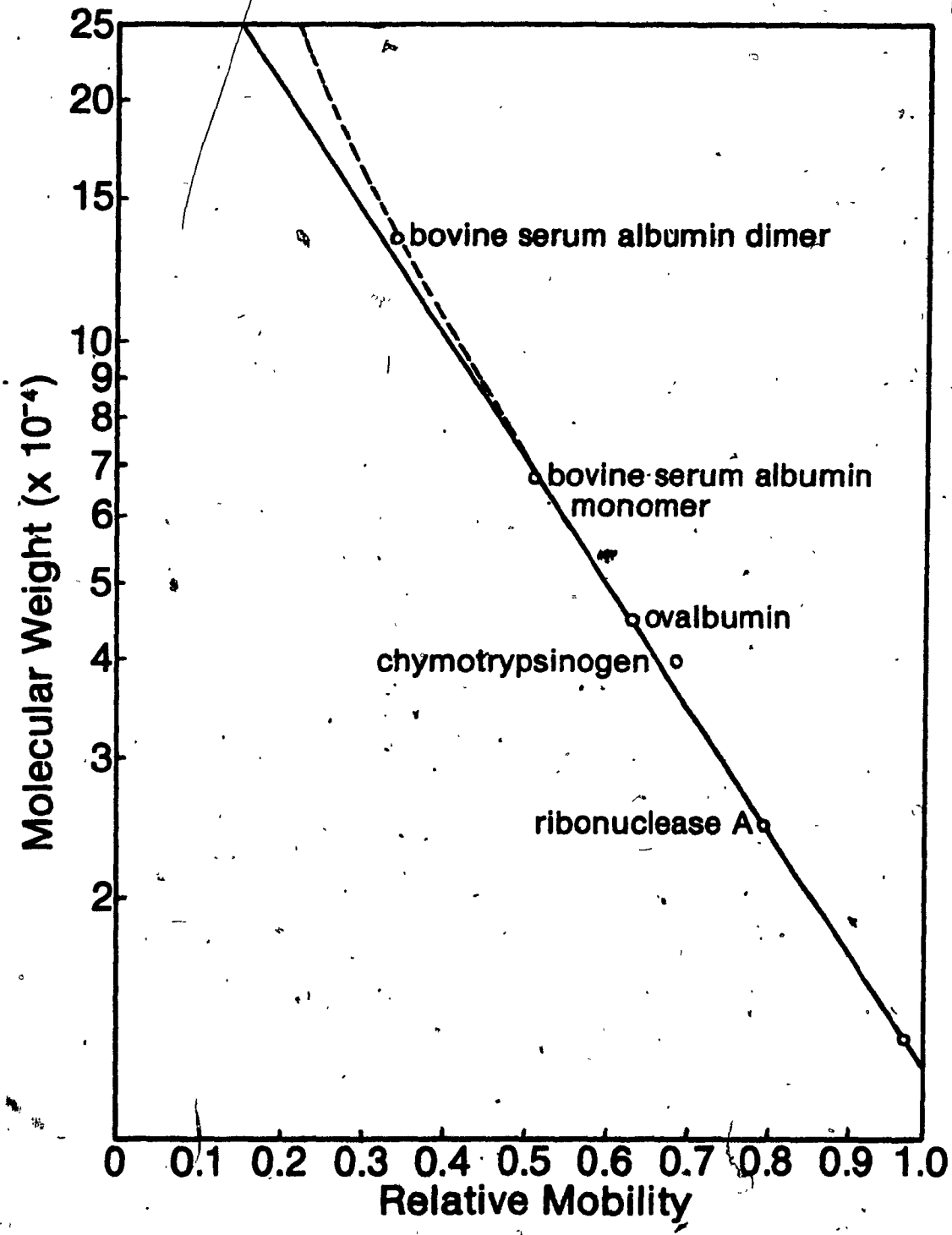


TABLE 19

SDS POLYACRYLAMIDE GELS ON STRIPPED MICROSOMAL MEMBRANE FROM 10 MELANOMA PATIENTS.

PATIENT	LENGTH OF GEL BEFORE STAINING	DISTANCE OF DYE MIGRATION	LENGTH OF GEL AFTER DESTAINING	DISTANCE OF PROTEIN MIGRATION
PER	11.3 cm.	9.3 cm	12.3 cm	2.0, 2.6, 3.3, 3.6, 4.4, 4.6, 5.3, 6.3, 7.5, 7.8, 8.3, 8.6, 9.1 cm.
VEN	11.6 cm	9.1 cm	12.6 cm	3,4, 4.0, 5.3, 6.3, 7.1, 7.3, 7.8, 8.2,8.8 cm.
MOR	11.4 cm	9.2 cm	12.5 cm	1,4, 2.0, 2.1, 2.6, 3.3, 3.6, 4.0, 4.4, 4.6, 5.4, 5.8, 6.1, 6.5, 6.8, 7.5., 7.9, 8.2,9.0 cm.
OLI	11.2 cm	9.5 cm	12.3 cm	1.9, 2.5, 3.8, 4.3, 4.6, 4.9, 5.4, 5.5, 6.3,

TABLE 19 CONTINUED

				6.5, 7.6, 8.1
				8.4, 8.9, 9.3 cm.
TRE	11.1 cm	9.1 cm	12.2 cm	1.4, 2.0, 2.6,
				3.3, 3.5, 4.0,
				4.4, 4.6, 5.2,
				5.6, 6.1, 6.7,
				7.5, 7.7, 8.1,
				8.4, 8.9 cm.
FEI	11.5 cm	9.4 cm	12.5 cm	2.1, 3.2, 3.5,
				4.3, 5.0, 5.5,
				6.0, 6.4, 6.9,
				7.4, 7.9, 8.3,
				8.4, 8.9 cm.
ZAI	11.4 cm	9.5 cm	12.5 cm	1.8, 2.5, 3.8,
				4.2, 4.6, 5.5,
				6.3, 6.5, 7.1,
				7.5, 8.1, 8.8,
				9.0, 9.3 cm.
SHU	11.4 cm	9.4 cm	12.5 cm	1.7, 3.2, 3.3,
				3.6, 4.4, 4.5,
				5.0, 5.5, 5.6,

TABLE 19 CONTINUED

				6.3, 6.5, 7.1, 7.6, 8.0, 8.4, 9.1 cm.
ROS	11.3 cm	9.5 cm	12.3 cm	2.1, 2.3, 2.6, 3.6, 4.0, 4.8, 5.3, 6.2, 6.5, 6.9, 7.5, 8.0, 8.9, 9.3 cm.
ELL	11.5 cm	9.6 cm	12.6 cm	2.2, 2.7, 3.6, 4.2, 5.6, 6.4, 7.4, 7.7, 8.2, 8.6, 9.2 cm.

TABLE 20

SDS PLYACYLAMIDE GELS ON MICROSOMAL ANTIGENS FROM MELANOMA PATIENTS USING AUTOLOGOUS SERA AS THE SOURCE OF ANTIBODY.

PATIENT	LENGTH OF GEL BEFORE STAINING	DISTANCE OF DYE MIGRATION	LENGTH OF GEL AFTER DESTAINING	DISTANCE OF PROTEIN MIGRATION
FEI	10.6 cm	9.7 cm	11.6 cm	4.5, 5.1, 5.6, 6.4, 7.0, 8.9 cm
ZAI	11.6 cm	10.3 cm	12.6 cm	5.8, 6.4, 6.9, 8.8 cm
SHU	12.0 cm	10.6 cm	13.1 cm	5.2, 6.1, 7.0, 7.6, 10.2 cm
ROS	10.9 cm	10.1 cm	11.9 cm	3.7, 4.9, 5.6, 6.5, 8.0 cm.

TABLE 21

SDS POLYACRYLAMIDE GEL ON (a) MICROSOMAL ANTIGENS ISOLATED FROM TWO MELANOMA PATIENTS USING ALLOGENEIC SERA AND (b) ANTIGEN(S) FROM NEGATIVE SERUM OF ONE MELANOMA PATIENT.

PATIENT	LENGTH OF GEL BEFORE STAINING	DISTANCE OF DYE MIGRATION	LENGTH OF GEL AFTER DESTAINING	DISTANCE OF PROTEIN MIGRATION
FEI *	11.8 cm	10.5 cm	12.8 cm	5.9 cm
ELL ‡	11.6 cm	10.3 cm	12.6 cm	5.8, 6.5 cm
FEI @	10.7 cm	10.0 cm	11.7 cm	5.7 cm

* Serum used for isolation from patient ELL

‡ Serum used for isolation from patient FEI

@ Negative serum used.

TABLE 22

MOBILITIES AND MOLECULAR WEIGHTS OF PROTEINS FROM MELANOMA PATIENTS MICROSOMAL MEMBRANE:

THE MOBILITY WAS CALCULATED AS -

$$\text{MOBILITY} = \frac{\text{DISTANCE OF PROTEIN MIGRATION}}{\text{LENGTH OF GEL AFTER DESTAINING}} \times \frac{\text{LENGTH OF GEL BEFORE STAINING}}{\text{DISTANCE OF DYE MIGRATION}}$$

CORRESPONDING MOLECULAR WEIGHTS OBTAINED FROM A PREPARED STANDARD CURVE.

PATIENT 1 PER

MOBILITY	CORRESPONDING MOLECULAR WEIGHTS	MOBILITY	CORRESPONDING MOLECULAR WEIGHTS
0.198	200 000	0.622	46 000
0.257	165 000	0.741	31 000
0.326	130 000	0.770	28 000
0.355	120 000	0.819	23 500
0.435	90 000	0.850	21 000
0.454	84 000	0.899	17 500
0.524	66 000		

PATIENT 2 VEN

0.344	125 000	0.739	32 000
0.405	100 000	0.789	26 000

TABLE 22 CONTINUED

0.536	62 000	0.830	22 000
0.627	46 000	0.890	18 000
0.718	33 500		

PATIENT 3 MOR

0.139	250 000	0.535	62 000
0.198	200 000	0.575	54 000
0.208	195 000	0.605	49 000
0.258	165 000	0.644	44 000
0.327	130 000	0.674	39 000
0.357	120 000	0.743	31 000
0.396	104 000	0.783	25 500
0.436	88 000	0.922	16 500
0.456	82 000		

PATIENT 4 OLI

0.182	220 000	0.604	50 000
0.240	180 000	0.623	47 000
0.364	120 000	0.728	32 000
0.412	100 000	0.776	26 500
0.441	88 000	0.805	24 000
0.470	79 000	0.853	21 000
0.518	67 000	0.891	18 000
0.527	64 000		

TABLE 22 CONTINUED

PATIENT 5 TRE

0.140	250 000	0.560	58 000
0.200	200 000	0.620	47 000
0.260	165 000	0.670	39 000
0.330	130 000	0.750	30 000
0.350	125 000	0.770	28 000
0.400	104 000	0.810	24 000
0.440	88 000	0.840	21 500
0.460	82 000	0.890	18 000
0.520	67 000		

TABLE 23

MOBILITIES AND MOLECULAR WEIGHTS OF PROTEINS FROM MELANOMA PATIENTS MICROSOMAL MEMBRANES AND ANTIGENS ISOLATED FROM SOLUBILIZED MICROSOMAL MEMBRANES USING AUTOLOGOUS SERA.

PATIENT 7 ZAI

MOBILITY	CORRESPONDING MOLECULAR WEIGHTS	
	MEMBRANE PROTEINS	ANTIGENS
0.173	230 000	
0.240	180 000	
0.365	115 000	

TABLE 23 CONTINUED

0.403	104 000	
0.442	88 000	
0.528	64 000	
0.528		64 000
0.563		58 000
0.605	49 000	
0.617		47 000
0.624	47 000	
0.682	38 000	
0.720	33 500	
0.768	28. 000	
0.786		26 000
0.845	21 000	
0.864	20 500	
0.892	18 000	

PATIENT 8 SHU

0.165	230 000
0.311	140 000
0.320	135 000
0.349	125 000
0.427	92 000

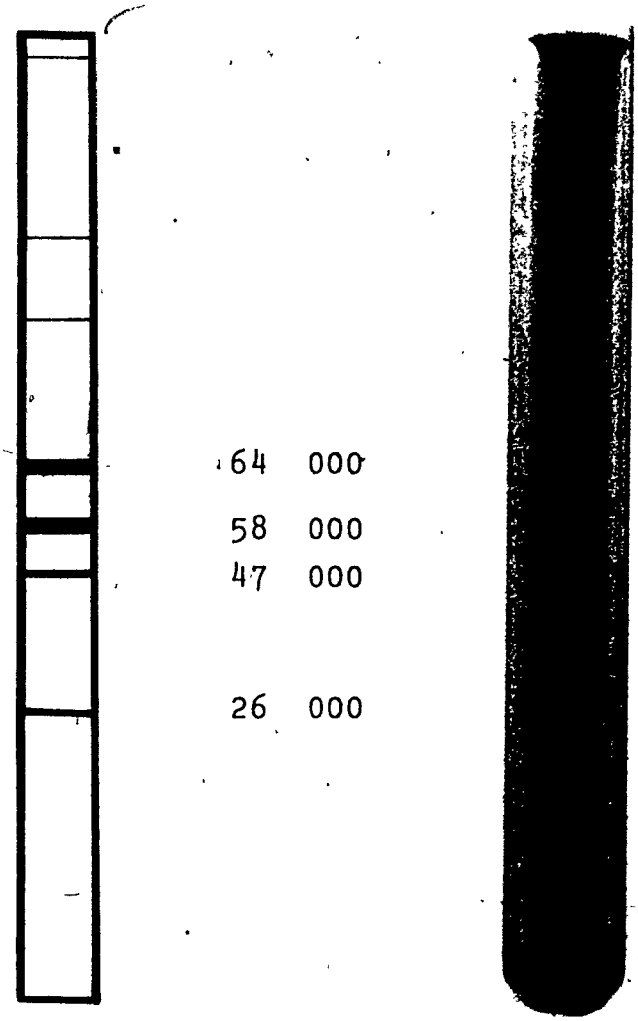


PLATE 4

Polyacrylamide gel electrophoresis of microsomal membrane antigens from melanoma patient ZAI. The microsomal membrane was solubilized with 1% Triton X 100 before applying onto the affinity column.

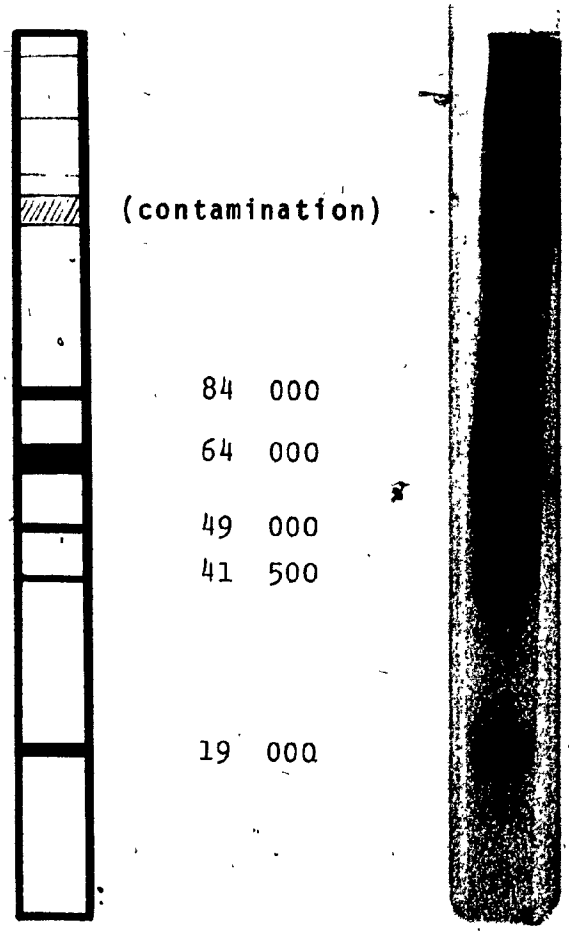


PLATE 5

Polyacrylamide gel electrophoresis of microsomal membrane antigens from melanoma patient 8 SHU. The microsomal membrane was solubilized with 1% Triton X 100 before applying onto the affinity column.

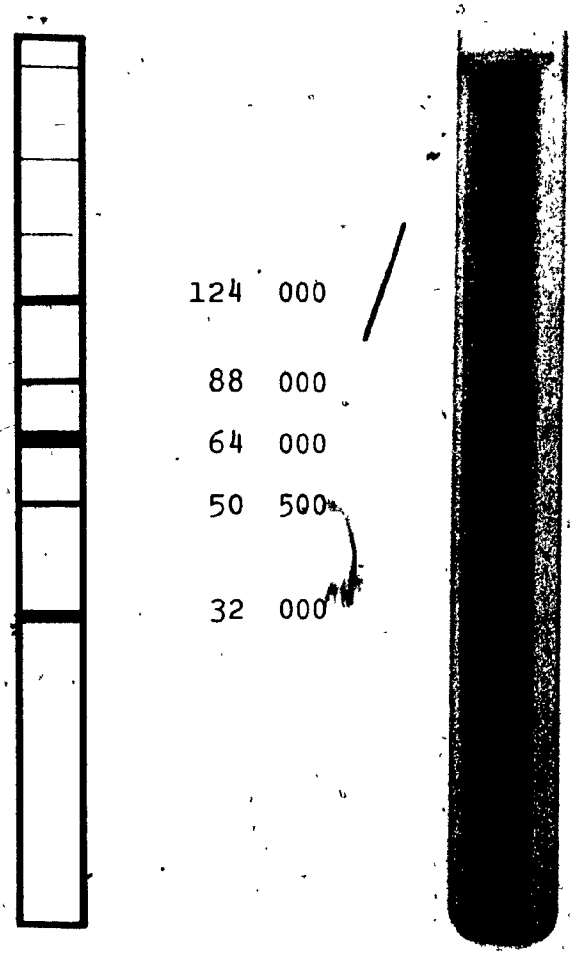


PLATE 6

Polyacrylamide gel electrophoresis of microsomal membrane antigens from melanoma patient 9 ROS. The microsomal membrane was solubilized with Triton X 100 before applying onto the affinity column.

TABLE 23 CONTINUED

0.446	84 000	
0.449		84 000
0.485	74 000	
0.527		64 000
0.534	64 000	
0.543	62 000	
0.605		49 000
0.611	49 000	
0.641	44 000	
0.656		41 500
0.688	37 000	
0.738	31 000	
0.776	26 500	
0.815	23 500	
0.880		19 000
0.883	19 000	

PATIENT 9 ROS

0.203	200 000	
0.222	190 000	
0.251	175 000	
0.335		124 000
0.348	125 000	
0.386	107 000	

TABLE 23 CONTINUED

0.444		88 000
0.464	82 000	
0.513	66 000	
0.526		64 000
0.598		50 500
0.599	50 500	
0.628	46 000	
0.667	39 000	
0.725	32 000	
0.725		32 000
0.773	28 000	
0.860	20 500	
0.899	17 500	

TABLE 24

MOBILITIES AND MOLECULAR WEIGHTS OF PROTEINS FROM A MELANOMA PATIENT MICROSOMAL MEMBRANE AND ANTIGENS ISOLATED FROM THE SOLUBILIZED MICROSOMAL MEMBRANE USING ALLOGENEIC SERUM.*

PATIENT 10 ELL

MOBILITY	CORRESPONDING MOLECULAR WEIGHTS	
	MEMBRANE PROTEINS	ANTIGENS
0.209	195 000	
0.256	165 000	
0.342	125 000	
0.399	104 000	
0.518		66 000
0.532	64 000	
0.581		54 000
0.609	49 000	
0.704	35 000	
0.732	32 000	
0.780	26 000	
0.818	23 000	
0.875	19 000	

* For this isolation IgG were obtained from positive serum of the patient FEI.

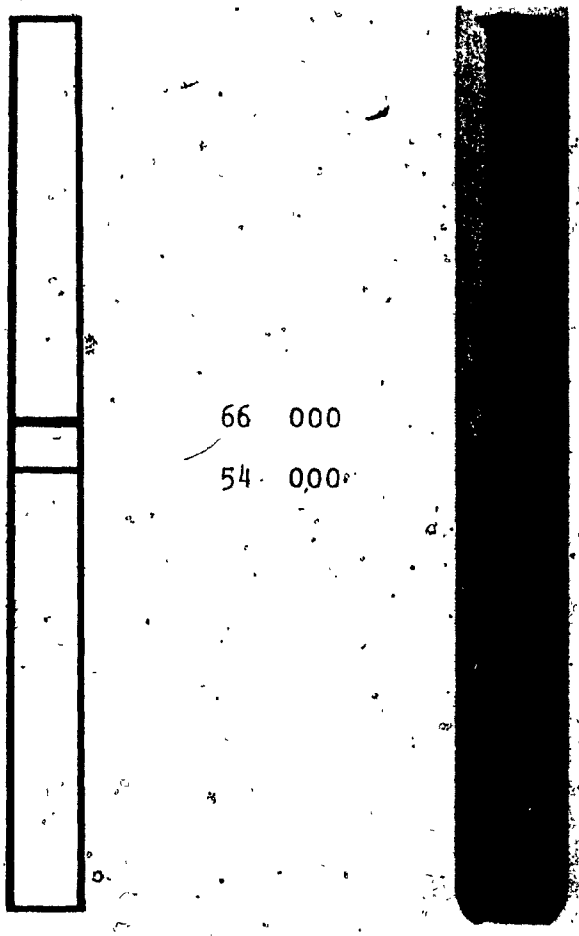


PLATE 7

Polyacrylamide gel electrophoresis of microsomal membrane antigens from melanoma patient 10 ELL. The microsomal membrane was solubilized with Triton X 100 before applying onto the affinity column.

TABLE 25

MOBILITIES AND MOLECULAR WEIGHTS OF PROTEINS FROM (a) A MELANOMA PATIENT MICROSOMAL MEMBRANE (b) ANTIGENS ISOLATED FROM THE SOLUBILIZED MICROSOMAL MEMBRANE USING AUTOLOGOUS SERUM (c) ALLOGENEIC SERUM AND (d) ANTIGEN(S) ISOLATED FROM AUTOLOGOUS NEGATIVE PATIENT SERUM CONTAINING ANTIGEN - ANTIBODY COMPLEXES.

PATIENT 6 FEI

MOBILITY	CORRESPONDING MOLECULAR WEIGHTS			
	MEMBRANE PROTEINS	MEMBRANE ANTIGENS*	MEMBRANE ANTIGENS‡	ANTIGEN(S) FROM COMPLEXES
0.205	195 000			
0.313	140 000			
0.342	124 000			
0.421	95 000			
0.424		95 000		
0.481		76 000		
0.589	74 000			
0.518				66 000
0.521			66 000	
0.528		64 000		
0.534	64 000			
0.587	52 500			
0.602	50 500			

TABLE 25 CONTINUED

0.603		50 500
0.660		41 500
0.675	38 000	
0.718	33 500	
0.773	28 000	
0.806	24 000	
0.815	23 500	
0.838		22 000
0.864	20 500	

* Antibody obtained from autologous sera

‡ Antibody obtained from allogeneic sera from the patient ELL.

PLATE 8

Polyacrylamide gel electrophoresis of microsomal membrane antigens (I and II) and antigen isolated from immune complexes (III) of negative serum from patient 6 FEI. The microsomal membrane was solubilized with Triton X 100 before applying onto the affinity column.

PLATE 9

Polyacrylamide gel electrophoresis of microsomal membranes from melanoma Patients PER, OLI, MOR, VEN and TRE.

— indicates antigen of molecular weight 50,000 ± 4,000

— indicates antigen of molecular weight 64,000 ± 3,000

PLATE 10

Polyacrylamide gel electrophoresis of microsomal membranes from melanoma Patients FEI, ZAI, SHU, ROS and ELL.

— indicates antigen of molecular weight 50,000 ± 4,000

— indicates antigen of molecular weight 64,000 ± 3,000

DISCUSSION

Previous investigations concerned with the identification and localization of tumor specific antigens (22, 24, 25) showed that there are mainly two groups of antigens localized in the cytoplasm of melanoma cells. These are (i) the autologous cytoplasm and (ii) allogeneic cytoplasm. These studies and others however did not elaborate as to which subcellular cytoplasmic organelle these antigens are associated with or give any detailed informations about the identification of these antigens, structurally and / or immunologically. The results of the present investigation thus furnish evidence regarding the localization of melanoma specific cytoplasmic antigens and additional biochemical and immunological informations are provided on these antigens which should bring us one step nearer towards an understanding of the immunological response and its subsequent failure in melanoma and possibly other types of metastatic diseases.

For this investigation ten malignant melanoma patients were studied (Table 1). Routine blood samples that were obtained from these patients to monitor the progress of the patient in response to the different forms of treatment administered were used as the source of antibody. Whenever

possible positive antitumor antibody serum, determined by cytoplasmic immunoflourescence, Tables 2, 3 and 4, was used to carry out the various immunological experiments. One current form of treatment involves the immunization of malignant melanoma patients with irradiated autologous tumor cells which were obtained from surgical operations on these patients. These excised tumors were in some cases large enough to provide excess tumor material which were used as the source of antigen in this investigation.

The results from Tables 5(a), 5(b) and 5(c) show that when melanoma cells from three melanoma patients PER, VEN and MOR respectively, were fractionated by the methods employed and six of the major subcellular fractions subjected to immunological analysis to determine the immunologically active fractions, there was consistency in the immunological pattern. Because of difficulty of diffusion of whole subcellular organelles in 1% Agar gels, positive immunoprecipitation was only observed for the Homogenate when these fractions were tested using the double diffusion Ouchterlony method. This was possibly due to the presence of free antigens sloughed off due to isolation procedures. More reliable results were obtained when the fractions were tested with countercurrent

immunoelectrophoresis on 1% Agarose gels. Strong immunoprecipitation was observed in all three cases for Band III, very weak immunoprecipitation in Band I and very weak precipitation line for Pellet 2 only in two patients, PER and MOR. Band I was not characterized but Pellet 2 is believed to contain whole nuclei. However our interest was centered around Band III, which by enzymatic assays (Tables 8(a) - 8(e)) and electron microscopy, was shown to be rough endoplasmic reticulum. The enzyme assay results indicate that for Oubain sensitive Na^+ / K^+ dependent ATPase (Table 8(a) and 8(b)) there seem to be an even distribution of this enzyme in both major subcellular fraction Band II and Band III. However, the results obtained from the Glucose 6 - Phosphatase (Table 8(c)) and the NADH Cytochrome c reductase (Table 8(d) and 8(e)) assays are more conclusive. Table 8(c) shows the highest specific activity for Glucose 6 - Phosphatase, an enzyme located mainly in the SER (51, 52), in Band II whereas Table 8 (e) shows the highest specific activity for NADH cytochrome c reductase, a marker enzyme for RER (51, 53), in Band III. Further fractionation of this fraction followed again by immunological testing of the resulting fractions led to in all cases, strong precipitation line in Band A III, Fraction I and Fraction II as shown in Tables 5(a) - 5(c).

Band A III which contained stripped rough endoplasmic reticulum membrane, characterized by chemical analysis such as protein, phospholipid and ribonucleic acid content PLP/RNA ratio (Tables 6(a) and 6(b)) was the fraction used for solubilization and isolation of antigen. 0.049% DOC treatment of this fraction from the above three patients resulted in the two immunologically active fractions, Fraction I and Fraction II. Fraction I was the reconstituted stripped RER membrane containing melanoma antigens within the membrane matrix and Fraction II contained the contents from the cisternal space of the stripped RER consisting of nascent melanoma antigens as well as other secretory proteins which were not immunologically active. This means that the nascent antigens synthesized on the ribosomes of the RER were vectorially discharged into the cisternal space of the RER via the RER membrane. Therefore it is reasonable to assume that the antigens located within the RER membrane are the same antigens localized within the cisternal space of the RER with minor structural modification. As a result further fractionation of Band A III with 0.049% DOC was not necessary and was not carried out on the stripped RER of the additional seven patients. For patients PER, VEN, MOR, OLI and TRE antigen isolations were not possible since only low quantities of

antigenic material were available for patient PER, VEN and MOR whereas highly positive sera were not available for patients OLI and TRE. However antigen isolations were accomplished for the five other patients used in this investigation.

Antigens were isolated from these patients when DEAE - cellulose partially purified IgG from highly positive sera were used to prepare Sepharose 4 B affinity columns. Tables 9(a) - 9(e), (Figures 4(a) - 4(e)), show the chromatographic pattern when 40% ammonium sulfate precipitated immunoglobulins from patients FEI, ZAI, SHU, ROS and ELL respectively were loaded onto DEAE cellulose columns and eluted with a NaCl gradient. In all cases IgG concentrations between 6 - 7 mg / ml (Table 9f) were obtained and this was used for coupling to CNBr activated Sepharose 4 B beads. Sonicated, 1% Triton X 100 solubilized microsomal membranes at the protein concentrations shown in Table 10 were then passed through separate columns and the bound antigen eluted with 3 M KSCN solution. Table 11(a) and 11(b) (Figures 5(a) and 5(b)) show the elution profile when solubilized microsomal membranes from patient FEI are eluted from affinity columns prepared with IgG obtained from autologous FEI and allogeneic ELL sera respectively. Tables 12 - 14 (Figures 6 - 8) show the elution profile for solubilized microsomal membranes

from patients ZAI, SHU and ROS, respectively when eluted from affinity columns prepared with IgG obtained from corresponding autologous serum. Table 15 (Figure 9) gives the elution profile for solubilized microsomal membrane from patient ELL when eluted from affinity columns prepared with IgG obtained from allogeneic serum from patient FEI. The outstanding feature of all these profiles is that in all cases the antigen was eluted in a single peak occuring in the first few fractions containing KSCN as shown in Table 16. Immunological tests on these fractions indicated that when they were tested against autologous serum strong immunoprecipitation lines were observed even when the amount of antigen was reduced to less than 5 ug with 1/4 dilution using Barbitone buffer pH 8.2 (Table 17). When these antigens were tested with allogeneic sera from different patients and control sera such as serum from sarcoma and normal patients, the pattern was less distinct. Weak or very weak precipitation lines were observed for all melanoma patients' sera except FEI II where strong precipitation lines were observed against ROS sera. No precipitation lines were observed with sera for the controls. This observation is consistent with the findings of Lewis (24, 28) who showed by cytoplasmic immunofluorescence the presence of group specific antigens in the cytoplasm of melanoma

cells which are capable of binding to positive sera from any melanoma patients. Antigen isolated from immune complexes in negative serum of patient FEI (Table 18) was also subjected to immunological analysis. Treatment of 50% ammonium sulfate precipitated protein with acidic buffer followed by molecular sieve chromatography yielded a peak that showed immunological activity against autologous positive serum but no activity against anti - human IgG. Again this is in good agreement with the observations of Lewis et al (32). These researchers found immunological activity between the IgG fraction of positive and negative sera whereas in this case the so called antibody - antigen (IgG - antigen) complexes were dissociated and the antibody and antigen were separated before immunological assays were done.

For structural analysis on these nascent cytoplasmic antigens SDS polyacrylamide gel electrophoresis were carried out on the antigens isolated from the five melanoma patients used as well as on stripped RER membrane of all ten patients studied (Tables 19 - 21). The solubilized antigens were applied onto 5.6% gels and the molecular weight of each protein band obtained by extrapolating the corresponding mobility on a standard curve (Figure 10). The molecular weights of the stripped RER membrane shown in Table 22 for patients PER, VEN, MOR, OLI and TRE respectively give a molecular weight range of proteins extending from 17,500

to 250,000 daltons. This molecular weight range has been obtained by other researchers (63, 77, 78) and it represents some 50 different species of polypeptide chains. The results from Table 23 demonstrate that for melanoma patient ZAI, nine nascent antigenic polypeptide chains can be isolated when autologous serum was used to prepare the affinity column. For patient SHU and ROS, ten nascent antigenic polypeptide chains were isolated in each case. In all three above patients two antigenic molecules are predominant; an antigen of molecular weight 64,000 $\pm$ 3,000 daltons is common in all three patients and is also found localized in the microsomal membranes of all ten patients studied. Another antigen with a molecular weight of 50,000 $\pm$ 4,000 daltons is also common in all three patients and can be localized, though less prominently, in the microsomal membranes of all patients. Similar results are seen in Table 24 which shows the molecular weights of antigens isolated when allogeneic serum from patient FEI was used to prepare the affinity column through which solubilized microsomal membrane from patient ELL was eluted. More interesting however are the results shown in Table 25. Here it is seen that the antigen of molecular weight 64,000 $\pm$ 3,000 daltons was isolated with autologous serum, with allogeneic serum from the patient ELL and from antibody - antigen complexes of negative serum

of the same patient that solubilized microsomal membranes were used to isolate antigens in the autologous and allogeneic cases (patient FEI).

From these results, two important observations emerge which could eventually play an important part in the understanding of melanoma antigens and particularly obtaining insight on antibody - antigen complexes and its relationship to the failure of the immune system in malignant melanoma. The first of these findings is that in melanoma there is a nascent antigen having a molecular weight of 64,000 $\pm$ 3,000 daltons which is believed to be allogeneic cytoplasmic antigen (melanoma specific antigen) and is localized on the RER membrane. Secondly, this antigen displays a structural relationship to that isolated from antibody - antigen complexes. Preliminary investigations (71, 79, 80) have shown that two antigenic molecules, one having a molecular weight of 50,000 $\pm$ 4,000 daltons and the other 12,000 $\pm$ 1,000 daltons can be isolated from antibody - antigen complexes. It has further been shown that when these two antigens are separated to give individual molecules and immunological test carried out using autologous and allogeneic sera, no immunoprecipitation was observed for both antigens

but when they were mixed and then tested immunologically, strong precipitation lines were observed with patient and cross - reacting sera. In light of this, the immediate question that has to be asked is whether the antigen having a molecular weight of 50,000 ± 4,000 daltons, which has been isolated from the solubilized melanoma patient's RER membrane and found present in the microsomal membrane of all ten patients studied is the same antigen having the molecular weight 64,000 ± 3,000 daltons. Here before the antigen is vectorially discharged into the cisternal space from the RER membrane the antigen matures further changing into a higher molecular weight antigenic molecule perhaps by the addition of some carbohydrate residues within the cisternal space of the RER.

Another interpretation could be that the 64,000 ± 3,000 daltons antigen comprises two antigens, 50,000 ± 4,000 daltons and 12,000 ± 1,000 daltons and that the low molecular weight antigen is a β_2- microglobulin as suggested by Thompson et al (64) and it combines with the higher molecular weight antigen before being recognized by the antibody to form the antibody - antigen complexes. At the moment it is unlikely that the real nature of these melanoma antigens can be elucidated with current knowledge of tumor antigens but hopefully, with the extensive research presently being carried in this and related fields, it should be possible to obtain a thorough

understanding of the role these antigens play in the staging of malignant melanoma.

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