IMMUNOELECTROPHORETIC ANALYSIS OF HUMAN MALIGNANT AND NORMAL TISSUES

by

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Introduction

Many attempts have been made to find an immunologically demonstrable difference between normal and malignant tissue. Since the literature in the field is voluminous, only a few representative papers concerned with different approaches to the subject have been reviewed in an attempt to indicate that some tumors differ antigenically from the normal tissue of origin.

It should be borne in mind that the neoplastic process may reflect a very small aberration in cellular function, whether it be the production of abnormal nucleic acids, nucleoproteins, enzymes, or other proteins. The methods available at present may be inadequate for the measurement of very small differences between tumors and other tissues.

Many theories of carcinogenesis have been presented in the literature. A brief presentation of a few of these seems pertinent since they may relate to demonstrable antigenic changes.

Osgood (52) suggests that the fundamental alteration is <u>any</u> genetic change leading to lack of any enzyme system necessary for a full life span of the differentiating cell of a series. The mature cells are thought to produce an inhibitor of cell division. The shortened life span, brought about by the genetic change, results in a reduction of the amount of inhibitor produced by the mature cells. This permits an increase in the mitotic rate of the immature cells of that series. Any increase in mitotic rate carries

with it the increased risk of further genetic change.

Green (24) proposes a theory of induction of neoplasia involving two stages: (1) precancerous hyperplasia without normal function or long continued stimulation without normal function leading to a local nutritional deficiency, then (2) if cells are present lacking some "identity protein" as a result of previous carcinogenic stimulation, they should tend to be selected for survival because they have fewer needs.

The first two theories presented imply a loss of something in the normal cell that may express itself as a loss of an antigen in tumor tissue. This would be very difficult to demonstrate by techniques used in the studies to be reported here, because it must be assumed that all tumors contain normal tissue as well. For this reason, controls should be normal tissues and serum, and would demonstrate unique antigens in the tumor, but would not demonstrate a loss of normal antigens.

At the present time no human tumors have been proved to be caused by viruses, though many are known in lower animals. Shope (54) has pointed out some of the difficulties encountered in applying a causative role to human viruses. Present knowledge leaves no conceptual barriers to the possibility that virus infections could be causative factors of cancer in any species (55). Bendich (3) has reported the isolation of deoxyribonucleic acid from polyoma virus-infected

cells which produces the same type of cytopathology as does the intact virus. The findings reveal that the infectious, carcinogenic principle of the virus is DNA, and that it is this which enters the cell to induce tumor formation.

This finding lends credence to the proposal by Goldthwait (20) that the cancer cell possesses an hereditary defect in the normal mechanism of control of cell division and since the nucleic acids form a chemical basis of heredity in the cell, it is reasonable to suspect that an alteration in them may be the basis for carcinogenesis. He presented evidence that some of the carcinogenic agents may affect DNA molecules. In the induction of tumors by RNA-virus, the virus may bring about the same alteration since RNA transmits information from DNA to the site of protein synthesis. A similar theory has been presented by Wakonig-Vaartaja (59).

The role of the host has often been overlooked when neoplasia is considered. In most definitions of neoplastic disease, one finds the phrase, "autonomous growth". This implies a steadily progressive process not influenced by its environment. Much evidence has been cited in the literature to suggest the existence of an immunological host response against tumor tissue. If this is so, then the presence of a unique antigen in tumor tissue is certainly probable. This is not to say that the antigen is the same, or even present, in all tumors.

Feldman (17) cites evidence that total body X-irradiation or administration of cortisone permits the progressive growth of microscopic tumor emboli, which normally reach the lymph nodes, but do not usually grow freely in these sites. Since both cortisone and X-irradiation are known to suppress the immune response, is such a course an indication of this mechanism? It has been observed by Krippaehne* that patients with no evidence of tumor for long periods of time following surgery for cancer, often appear with widely disseminated disease within a few months after serious emotional circumstances, such as the death of a spouse.

Graham and Graham (23) have presented data on complement fixing antibodies in the sera of patients with various gynecologic cancers. In some cases there was no detectable titer before the excision of the tumor, but antibody appeared after removal. This may have indicated the <u>in vivo</u> absorption of these antibodies by the tumor. The tumor extract used in this work was water soluble and saline insoluble.

Baruah (1) has observed many tumors with lymphoid and plasma cell proliferation in surrounding tissue.

Lovell (40) has reported a case of "spontaneous regression" of metastatic carcinoma of the colon in which a hyperplastic cervical node was found during a biopsy. This is interesting in view of the work of Black, et al. (6,7,8,9,10), who have devised a grading system in which

*Krippaehne, Personal Communication. 1962.

the results correlate linearly with prognosis and survival. They find that, of the three features which they evaluate, the presence of a lymphocytic infiltrate in the primary tumor, or a sinus histiocytosis in the regional nodes, usually allows for significant improvement in prognosis and survival.

Everson and Cole (16) have reviewed 600 reported cases of tumor regression. Of these, 112 were well-documented cases of regression of histologically proved malignant disease. Most of these occurred in relatively rare types of tumors. This fact has led the writer to wonder if these types occur more commonly, but are destroyed early by body defenses, before clinical expression of the disease is present.

In the past two decades, much work has been done to determine if the serum of cancer patients contains an antigen not found in normal serum. Some of the first studies (46,47) consisted of immunizing rabbits with human tumor tissue and using the antiserum in a precipitin test against the serum of patients with cancer. Though the experimental controls were less than adequate, the results were suggestive that specific antigens were present in the sera.

Another method of approach to this problem has been the use of the Schultz-Dale bath (11,12,42,44). In this procedure, a virgin guinea pig was immunized with tumor and Freund's adjuvant. After 4 weeks, the animal was sacrificed and the uterine horns (which now contained

antibody) were suspended in a physiological saline bath. For controls, unsensitized uterine horns were used. Both had been desensitized to normal human plasma before the experiment began. Serum was then added to each bath. If a serum caused a contraction in the sensitized horn and not in the unsensitized one, it was considered positive. Only contractions which were one-fifth as strong as those produced by 10 gamma of histamine were considered positive. In very few cases were the results equivocal, the contraction usually being much greater or much less than the arbitrary value. One series (12) consisted of 301 cases of histologically proved carcinoma and 207 non-carcinomatous patients. In both groups, the correct diagnosis was made in 97% of the patients. Similar results were obtained in other series. There is evidence to suggest that the antigen detected by this method is a polysaccharide (13).

Darcy (14) found it possible to distinguish the plasma of rats bearing transplanted or induced tumors from that of normal rats or rats bearing homografts of normal tissues. He immunized rabbits with plasma or serum of normal rats and of those bearing transplanted Walker tumor. In gel diffusion plates, when reacting tumor-plasma against antisera to normal plasma, he consistently found the albumin arc more distant from the antiserum reservoir than when he reacted normal plasma against the same antiserum. Another line, unidentified, also showed this characteristic.

Two smaller arcs situated near the antibody reservoir were consistently more prominent in the tumor-plasma zone. With antiserum to the tumor-plasma, he obtained the same results. In addition, the tumor-plasma, when reacting with antitumor-plasma serum, resulted in an arc which was entirely absent from the reaction of the same antiserum with normal plasma. The position of the albumin arc is consistent with the statement of Hauschka (27) that albumin is found in smaller concentrations in the blood of tumor-bearing animals.

Hirsch, et al. (29), utilizing well in-bred strains of mice and spontaneous tumors of recent origin, found that iso-immunization resulted in no difference in the total number of tumors or in the time of appearance of the tumors. However, there was an increase in survival time in the immunized group. They concluded that, "the in-bred mouse can be immunized against tumors of its own strain of recent origin, but that the extent of immunity thus achieved is a rather small and subtle one".

Hauschka (27) cites an experiment whereby a tumor antiserum was prepared by inoculating rabbits with centrifugates of C3H mammary tumors, cell suspensions of which were later incubated with the immune serum for several hours and then tested for carcinogenic activity in ZBC mice. Tumor growth was completely forestalled by the incubation with the antiserum, while cancer controls incubated in saline

rapidly developed into fatal neoplasms in all inoculated animals. Further controls incubated with antiserum toward normal mammary tissue, or with ordinary rabbit serum, also developed transplant tumors, but at a slower rate.

Wigzell (61) reports that the intraperitoneal injection of lymphoid cells of one parental strain into F1 hybrid mice inhibits the growth of lymphoma cells of the opposite parental strain inoculated subcutaneously. He suggests that this is accomplished by the release of a humoral antibody.

In another cytotoxicity study, Toolan and Wallace (58) reported that immune serum to HEp #3 tumor cells could inhibit their growth in culture, but normal tissue antiserum could not. They found the responsible antigenicity in an insoluble part of the tumor cells and suggested it may be in the cell membrane.

Southam, et al. (56) found that injections of viable human cancer cells in healthy humans resulted in accelerated destruction of subsequently injected cancer cells of the same or other type. In preliminary experiments, pretreatment with normal amnion cells or fibroblasts did not accelerate the destruction of subsequently injected cancer cells.

Lomakin (39) has demonstrated cytotoxicity of tumor DNP antiserum in tissue culture. He felt that the effect was greater on the tumor cell cultures than on those of

homologous normal tissue. Many details of the work are lacking in his report, therefore his conclusions cannot be evaluated satisfactorily.

Some investigators have attempted to immunize patients against their own tumors. Witebsky, Rose, and Shulman (63) have emulified thyroid carcinomas in Freund's adjuvant and injected the emulsion into the donors. Results of this procedure were not encouraging. However, all of the patients were in the terminal stage of their disease, and the authors stated that similar attempts at an earlier stage might produce different findings. Better results with this same procedure have been cited by Milgrom (50).

Using complement fixation, Kidd (33) made a detailed study of a special antigen in saline extracts of the Brown-Pearce rabbit carcinoma. The tumor extracts fixed the complement with sera of rabbits of the blue cross strain implanted with this tumor. No reaction was obtained with extracts from tissues of normal kidney, liver, spleen, red bone marrow, pus, or other rabbit tumors. He thought the active factor was a ribonucleoprotein.

Zilber (65,66), using gel diffusion analysis on a transplantable hepatoma of mice, found there was a loss of two antigens in the tumor tissue, one of which appeared to be liver-specific. He found also that there was one antigen in the tumor tissue that did not occur in normal liver. After absorption of the hepatoma antiserum with

normal liver antigens, the precipitate in the gel persisted and even became more pronounced. He suggested that this antigen was tumor-specific. This view was supported by experiments involving anaphylactic reactions in animals after desensitization with normal liver. An antigen was also found by inducing "immunological tolerance" in rats to normal liver tissue, then immunizing with the hepatoma. It would be interesting to know if such a serum reacted with the same antigen found in the gel diffusion procedure. To my knowledge, no statement has been made in this regard.

Bjorklund (4) immunized a horse with 56 pooled human tumors. Using gel diffusion, he analysed the tumor pool, normal plasma, and 16 normal human tissues. Four cellular antigens were found, none of which were specific to the tumor pool. One was found in liver, spleen, lung, kidney and brain but not in eleven other normal tissues. The other three were generally distributed. It was also reported (5) that the pooled-tumor antiserum was cytotoxic for monolayer cultures of HeLa cells. This toxicity was no longer present after absorption of the serum by human carcinomas. It was not stated whether absorption with normal tissue was attempted.

Kosyakov and Korostelova (38), using complement fixation and absorption of antisera with normal tissue and serum, have reported finding tumor-specific antigens. Some tumors were said to contain qualitatively similar antigens while

others appeared to be unique. The location of the primary neoplasm did not appear to have a relationship to the immunological grouping. Metastatic lesions were not found to differ qualitatively from the primary tumor. Quantitative differences between the primary and metastatic lesion have been demonstrated by histochemical staining (45).

There have been some reports of an organ-specific antigen loss in tumor tissue. Using rat hepatomas, hamster renal carcinomas, human skin cancer and the homologous normal tissue of each, Nairn (51) prepared a suspension that was rich in cytoplasmic particles derived from the endoplasmic reticulum. He used complement fixation, gel diffusion and fluorescent staining to show that the three normal tissues reacted with their homologous antiserum. One precipitate remained in the gel after absorption with many tissues, including the malignant tissue of the same organ. Only the normal tissue of origin could absorb the antibodies such that the one precipitate would not appear. Weiler (60) also reports a similar loss of antigen in tumor tissue. These findings are in agreement with Green's (24) theory of carcinogenesis described previously.

De Carvalho (15) has used homogenization of malignant cells with a fluorocarbon (Genetron 113) to separate some of the normal antigens from the tissues. After preparation of the tumor extract, normal antigens were absorbed with rabbit antiserum to human amnion cells. Gel diffusion

techniques were applied to evaluate the tumor antigens. He found that the specific antigens present in solid tumors were different from the ones found in leukemia cells and, within the leukemias, there were different antigens in acute stem cell and in chronic lymphatic leukemias. No differences were demonstrated between carcinomas and sarcomas.

Metzgar, et al. (49) have studied a rhesus monkey histiocytoma which was virus-caused. Genetron extraction of the tumor allowed for a virus suspension with fewer tissue proteins. Repeated extractions with Genetron 113 resulted in loss of reaction to complement fixation and a longer latent period for tumor induction when the cell-free material was reinjected.

After Genetron treatment, Taylor, et al. (57) have demonstrated specific complement fixing antigens in the following tumors: (1) H. Ep. No. 2, (2) human sarcoma, (3) germ-free chick tumor, and (4) Rous sarcoma (which has a known viral etiology). All complement fixing antigen was removed from normal germ-free chick muscle. It was suggested that Genetron treatment of tumors be used to remove normal tissue materials from tumor suspensions before immunological analysis. Also, it was proposed that this method be used to isolate viruses or virus-like material from tumor tissue in which a viral etiology has not been established.

McKenna, et al. (48) have used the same extraction method on HeLa cells, J111 human leukemic cells and normal human uterus. There was no cross reactivity between the two neoplastic cell lines as determined by complement fixation. The normal uterus extract contained no protein detectable by precipitation with trichloroacetic acid, yet rabbits were immunized with this solution. There was no reaction between it and its homologous antiserum in complement fixation tests. Apparently all normal antigens were removed during the extraction process. Using the tanned red cell hemagglutination technique, they state, "We sensitized red cells with the product obtained by Genetron treatment of HeLa cells, or with normal human uterus treated in the same way. A total of 366 human sera have been assayed by this technique. Of the 193 sera from patients with various malignancies, 49 (25 percent) showed evidence of antibody capable of reacting with the HeLa extract, and nine (5 percent) other sera reacted with the J111 extract. None of the sera from patients with malignancy reacted with the uterine material and none of the remaining 173 sera from patients without malignancy reacted with extracts of HeLa, J111, or uterus. Of 49 patients whose sera showed evidence of antibody to HeLa extract, 75 percent were from patients with known metastatic disease."

The preceding investigations and observations lend credence to the idea that immunologically detectable

changes often occur in the neoplastic process. With this in mind, various studies with immunoelectrophoresis to be reported here have been carried out.

Materials and Methods

Tissues

The normal and malignant tissues used in this work are from three sources: (1) surgical specimens, (2) autopsy specimens, and (3) tissue culture.

The surgical specimens were obtained in the surgical suite immediately after removal from the patient. They were transported to the laboratory in sterile beakers, and cleaned free of necrotic tissue and adjacent connective tissue. Some were extracted immediately and others were frozen in the refrigerator for later use.

Necropsy tissues were received within 12 hours of the death of the patient, and were handled in the same way as the surgical specimens. No tissues were accepted from embalmed cadavers.

One strain of tissue culture cells was used. J111 human leukemic cells from the Department of Experimental Medicine were obtained from tissue culture bottles where they had been grown in M. E. M. medium. The suspensions were centrifuged at 3000 x g for 15 minutes. An estimated 500 milligrams of cells were obtained from this source. They were washed three times in 0.1 M saline, and Genetron and distilled water extracts were prepared in a manner that will be described subsequently.

Antigen Preparation (Fig. 1)

Saline extracts. Tissues were cut into small pieces with a scalpel. Nine cc. of 0.1 M saline was added for each gram of tissue. This mixture was homogenized at $4^{o}C.$ in a

Vir-Tis 45 homogenizer at high speed for 5 minutes. Initially, some of the mixture was put on a slide and stained with Methylene Blue. Microscopic examination of these slides indicated that the cells had been adequately destroyed. After it had been demonstrated that the homogenization procedure was satisfactory, the microscopic checking was abandoned. The preparation was stored in the refrigerator for 4 to 8 hours, with occasional stirring, then centrifuged at 10,000 x g for 30 minutes at 5°C. in an International Model HR-1 centrifuge. The supernatant was then concentrated to one-half its original volume by dialyzing against a highly concentrated solution of pyrogallol ("Carbowax" 20M of Union Carbide Chemicals Corporation), according to the method of Kohn (34). This final extract was stored in sterile serum bottles with tightly fitting rubber caps. Most were kept in the freezing compartment of the refrigerator. It seemed best to store them in small aliquots so that once each had been used, it might be discarded. This obviated refreezing which would have increased the chances of protein destruction.

Later saline extracts were made as described above except they were centrifuged at 3000 x g for 15 minutes and dialysis was not performed.

Distilled water extracts. The tissues were weighed and 5 cc. of double distilled water was added for each gram of wet tissue. These were homogenized at 4°C. for 5 minutes at high speed in a Vir-Tis 45 homogenizer. After standing

in the refrigerator with constant stirring by a magnetic stirrer for one-half hour, they were centrifuged at 3000 x g at 4°C. for 10 minutes. The supernatants were collected and the extracts were designated as crude or "C", antigens.

Genetron extracts. These were prepared by the method of McKenna, et.al. (48). An aliquot of the distilled water extract described above was placed in the homogenizer at 4°C. One-half volume of Genetron 113 (Trichlorotrifluoroethane) was added. This was mixed in the homogenizer for 1 minute at high speed. Each Genetron-treated extract was centrifuged at 3000 x g at 4°C for 10 minutes and the supernatant was saved. This process was repeated with the supernatant for a total of five extractions. The final extract was designated Genetron, or "G", antigen.

Deoxyribonucleoprotein. This antigen was prepared and made available by Dr. Richard B. Lyons of the Department of Anatomy. The tissue used in its preparation was the carcinoma of the kidney from which antigens AgKC, AgKG5, and AgKS were prepared. This was a surgical specimen in which the tumor was well demarcated and located on the lower pole of the left kidney. For this reason, normal kidney was obtainable from the upper pole. No malignant cells were seen in this area on histological sections stained with hematoxylin and eosin. Also, the tumor area revealed no evidence of venous or lymphatic invasion. DNP

was prepared from the normal portion of this same kidney. The other antigens prepared from the normal tissue were designated AgKNC, AgKNG5, and AgKNS.

Antisera

Inoculations. New Zealand white rabbits weighing 5 to 7 pounds were used to form the antisera. Rabbits are considered to be among the best precipitin forming animals (32). For each injection, 1.25 cc. of the antigen preparation and 1.25 cc. of Freund's (18,19) Adjuvant-Complete (Difco) were placed in a 3 cc. serum bottle. The bottle was then attached to a Wig-L-Bug Amalgamator and vibrated for 2 to 4 minutes until the solutions were well emulsified. Emulsification was considered complete and the material ready for injection if a drop did not disperse when placed on the surface of water in a beaker. Each rabbit was injected with 1.5 cc. of the emulsion and this was repeated 2 weeks later, using a freshly prepared inoculum. At first, all injections were made subcutaneously. This was later changed to intramuscular injections with no qualitative alteration in antibody response. The change was made because about 1 out of every 10 subcutaneous injections produced sterile abscesses, whereas none have appeared at the intramuscular sites. It is felt that the better vascular supply of the muscle may have played a role in this phenomenon.

Bleeding. The rabbits were confined in a box designed to allow little if any movement. The box could be arranged so that either the animal's entire head was outside the box, or the ears only. The hair was trimmed as closely as possible with a pair of scissors within a radius of two centimeters around a point over the marginal ear vein. The area was then swabbed with 70% ethanol and dried with sterile cotton. A Bard-Parker #11 scalpel blade was used to incise the vein longitudinally for 2 to 3 millimeters. The blood was then allowed to drip into a sterile test tube. The velocity of flow increased markedly if heat was applied to the underside of the ear. This was accomplished by holding a stainless steel centrifuge tube filled with hot water against the lower surface of the ear. This did not promote hemolysis of red blood cells; indeed, it was the impression that less hemolysis occurred in samples so obtained. The tubes of blood were closed with Parafilm. After the blood had been allowed to clot and retract for 4 hours at room temperature, the serum was collected and centrifuged at 1000 x g for 10 to 15 minutes. It was stored frozen in the same way as the antigens.

Bleeding was usually done 2 to 4 weeks after the last injection. Most investigators using gel diffusion techniques disregard titers because the results are dependent on the qualitative differences in components of the serum.

Gamma globulin precipitation. This was accomplished by the method of Heidelberger, et. al. (28) using a filtered solution of sodium sulfate saturated at 35 to 38°C. as the precipitating agent. The serum was stored in the refrigerator for one week, then freed from separated lipids and sediment. An equal volume of sodium sulfate was added to the serum at 37°C. After centrifugation at 1500 x g for 20 minutes, the supernatant was discarded. The precipitate was broken up and recentrifuged until no more liquid could be obtained. The pellet was dissolved in distilled water, filtered and dialyzed against 0.1 M saline for 24 hours. Paper electrophoresis with subsequent staining and scanning was performed by the Clinical Pathology Department.

Immunoelectrophoretic Analysis

IEA has become a very useful and popular investigative tool. The method and theory involved is thoroughly reviewed in the literature (13,21,22,30,35,41,53,62,64), and should not need repeating here.

Agar. A 1% solution of Difco Bacto-agar was prepared using veronal buffer, pH 8.6 and ionic strength of 0.0375. If particulate materials were present, the solution was centrifuged while still hot. This procedure was usually unnecessary. An 8 x 12 inch Pyrex dish was partially filled with hot agar. When the gel formed, it provided a level plane on which eight 2 x 3 inch glass slides were placed. Hot agar was then poured on the slides until it

was 3 mm. deep. Stainless steel siliconized dies (Fig.2c) for making antigen wells and antiserum troughs were placed on the glass slides and the agar was allowed to cool. The forms were removed and the slides were cut out of the agar with a scalpel, leaving a 3 mm. layer on the top surface, with appropriately spaced wells and troughs (Fig.2b). It is important that the agar be even with the edge of the slide because the anode and cathode wicks of the electrophoresis cell must make good contact with the edge of the agar. Preservatives were not used because they have been known to interfere with precipitation (41).

<u>Plates</u>. The 2 x 3 inch glass slides were thoroughly washed with detergent, rinsed in distilled water, and labelled on the underside with a diamond pencil to designate the cathode and anode ends. One ml. of the appropriate hot agar solution was placed on the slide and spread evenly over the entire surface. These were placed in a hot oven for 15 minutes. They were then stored in sets of 8 in aluminum foil. This thin layer of agar provides better contact between the agar and the glass when the final plates are made and probably prevents some capillary flow of antigen and antiserum at the glass-agar interface when the wells and troughs are filled. The final steps in the preparation of the plates has been discussed previously

<u>Filling of the plates</u>. Tuberculin syringes and #24 gauge needles were used to deliver reactants to the

appropriate reservoirs. The 3 mm. diameter wells were filled with 0.01 cc. of antigen extract. The 1 x 38 mm. troughs were filled with 0.1 cc. of antiserum.

Electrophoresis. A standard paper electrophoresis apparatus (Spinco Durrum cell and Duostat) was employed (Fig. 2d). Each cell was filled with 1 liter of the same veronal buffer that was used to make the agar. The Durrum cell was set up in the same way as for paper electrophoresis, except that the paper strips and their holders were omitted. The agar plates were placed lengthwise between the negative and positive wicks. The wicks were then pressed against the edge of the agar on each plate. It is important that good contact be made between all exposed agar edges and the wicks. If everything has been done correctly, the Duostat can be turned on to any constant voltage and the amperage should be distributed equally between the two cells. The antigen wells were then filled as previously described and the current was immediately applied in order to prevent diffusion toward the troughs. A constant voltage of 70 to 90 volts was used in this work. This usually resulted in about 4 mAmps. per plate. Three to 5 hours was found to be adequate time for good separation in nearly all plates. At the end of the period of electrophoresis, the current was turned off and the antibody troughs were filled in a manner described previously. The plates were developed in

the humid atmosphere of the Durrum cell or in a specially constructed plastic moist chamber for one to three days.

Cellulose acetate electrophoresis. This was performed once using antigens AgKC and AgKG5. Cellulose Acetate Strips (Oxoid) were cut to pieces 1 x 4 inches. The smallest amount of solution that could possibly be applied was placed across the center of the strip with a capillary pipette. Each end of the strip was placed between a wick and its respective holder. A constant current of 2 mAmps. per strip was then applied for 3 hours. The strips were carefully removed and fixed in the oven at 50°C. for 1 hour. These were stained for protein and deoxyribonucleic acid by Miss Dorothy Corey in the Department of Anatomy.

Photography. Dark background illumination of the plates was provided by a box lined with aluminum foil, containing two 100 watt bulbs and having a piece of black felt on an asbestos base supported above the bulbs (Fig.2a). The diffuse light passed through an aperture on top of the box over which the agar plate was placed. A Miranda D and an Exacta 35mm. reflex camera have both been used with good results. Kodak High Contrast Copy film and Kodak D-11 developer were used (31,41). Pictures were taken at least once a day for 3 days. Photographing each plate at least twice in the first 24 hours was found to be desirable. The film was developed immediately after photographing to prevent loss of records of the plates if the film processing

was done incorrectly. It also allowed the investigator to compare the negatives with the plates to assure that all precipitation arcs were visible on the film. Using these photographic methods and materials it was unusual for an arc to be absent on the film when it was visible on the plate. Indeed, it was sometimes the case that an arc was clearly visible on the negative when it was difficult to visualize on the agar plate. This finding confirms that of Crowle (13) and Lyons (41).

Staining of plates. After 3 days, the plates were washed for 24 hours in saline to remove materials that were not involved in the antigen-antibody precipitation. They were washed in 3% acetic acid for 2 hours, rinsed in distilled water, and dried by placing a wet filter paper on top and allowing evaporation to occur. The precipitates were stained with Amidoschwarz 10B (Buffalo Black) which has been found to be a good stain for rabbit precipitins.

Records of results. Agar plates can be allowed to dry as previously described, then stored in a filing system. These can be rehydrated at a later date. This was not found to be necessary since observation and photography provided a complete picture of the original reactions. A photographic enlarger was used to compile results. The negative was projected on a heavy piece of white paper and the arcs were traced. The negative was then compared with the tracing in direct light, and any additional arcs observed were drawn into the tracing.

Results

The technique used in this work requires that a special method be used to present the results. In the first place, many reactions must be run to find the most appropriate antiserum to be used from the animals that have been immunized with a certain antigen. Also, each reaction requires several observations at intervals throughout a period of time, since arcs appear at different times and some arcs disappear before the plate is mature. A plate is considered mature when no new arcs appear. This is usually by the end of the third day. The results have been summarized by compiling data from many reactions in such a way that temporal relationships are not taken into account (Fig.3). When this has appeared to be of importance, it is discussed in the text. Each capital letter preceding a set of reactions designates the antiserum used in the trough. The lettering just below each reaction zone designates the antigen in the reservoir (Table 1). In the reactions between an antigen and its homologous antiserum, the arcs have been lettered to facilitate discussion in the text. The lettering of arcs in one homologous reaction does not relate to the designations given to arcs in other homologous reactions. The code employed for the antiserum is explained in the legend of Figure 3. For example, A in Figure 3 denotes the antiserum AbK2a. The homologous reaction is with the antigen AgK2a.

The prefix Ag (meaning "antigen") has been omitted from the figure. The antiserum to AgK2a is labelled AbK2a. This designation holds throughout the paper except that some human sera have been used as antigens and antisera. In these cases, the labelling is the same for antigen and antiserum and a description of the source is presented in the antigen summary (Table 1).

Antigenic Analysis

The results of the analysis of antigens are presented below by considering the reaction between one antiserum and each of the reactions in which it was involved. A specific reference to an arc refers to the corresponding arc in the homologous reaction of this particular antiserum unless otherwise stated.

AbK2a (Series A in Fig. 3). The homologous reaction between AbK2a and AgK2a demonstrated a minimum of 5 antigens in the tumor extract (labelled a to e). There was only slight electrophoretic migration of any of these antigens, c showing the greatest migration toward the cathode and b the greatest anodal movement. The reaction of this same antiserum with the normal kidney extract (K2b) demonstrates all of the antigens seen in the tumor except for e. A macroglobulin was obtained from the serum of this patient (K2c) and was used as an antigen against AbK2a and K2b. Dr. Rigas stated that its migration was with the gamma globulin fraction of the serum, and it demonstrated a

similar configuration as that previously reported (37). Antigens b and c were demonstrated in extracts P7a, P7b, Br6, K8a, K8b, K8c, and CML4a. Antigen d was also present in Br6, K8a, K8b and CML4a. Arc c was the only demonstrable antigen in Par3 and CML4b.

AbK2b (Series B). Using this antiserum, there are 4 demonstrable antigens in the homologous reaction. The same 4 antigens are demonstrable in the reaction with AgK2a. These antigens correspond to b, c, d, and e in the homologous reaction of AbK2a. Arc a is not present. This antiserum reacted in the manner previously described with AgK2c. P7a and P7b contain three antigens that correspond to arcs a, b, and c. Br6 contains at least 4 common antigens, 3 of which correspond to arcs b, c, and d. The other one is probably a, although this is not certain. K8a demonstrates arcs b, c, and d, whereas K8b and K8c contain another antigen that does not appear to be the same as any demonstrated in the homologous reaction. CML4a shows a similar reaction as K8b and K8c.

AbP7a (Series C). The homologous reaction contains a minimum of 3 antigens, a migrating strongly to the cathode, b migrating slightly to the cathode, and c migrating strongly to the anode. P7b contains these three antigens plus another one on the anode side of the plate. Arc b was the only one demonstrated in K2a and K2b. CML4a demonstrates all three antigens but CML4b shows only a and b. Br6 demonstrated

only b and c. Gotham Serum contains a and b and also gave another arc on the cathode side of the antigen well which is not demonstrated in the homologous reaction. This is also the situation with Buch. Serum #2, which demonstrates an arc near the antigen well that is not represented in the homologous reaction. Buch. Serum #1 showed a and b.

AbP7b (Series D). This homologous reaction contained a minimum of 5 antigens. Arc c, which has the greatest cathodal migration is diphasic. This indicates a group of substances with similar immunological properties, but having different electrophoretic mobilities and is characteristic of gamma globulins. Arcs d, e, and either b or c are present in P7a. K2a and K2b are similar to P7a, except that K2b contains an anodic precipitate not seen in the homologous reaction. Though difficult to ascertain with certainty, that arc may represent an extension of the diphasic gamma globulin. This phenomenon is seen more clearly in Br6. CML4a, CML4b, and Br6 are similar and present arcs b, c, d, and e. Gotham Serum and Buch Serum #2 demonstrate antigens b, c, and d.

AbBr6 (Series E). AgBr6 is seen to contain a minimum of 4 antigens, one (a) with a strong affinity for the cathode and the other three with only slight migration. The three human sera examined with this antiserum were uniform in that all arcs were represented except b.

AbCML4a (Series F). It is of interest that Buch. Serum #1 demonstrates only three arcs against this antiserum, whereas Buch. Serum #2 and Gotham Serum have an additional precipitate near the antibody trough which has virtually no migration. Note that Buch. Serum #1 was collected prior to surgery. The possible significance of this observation will be discussed subsequently. The remaining 3 arcs are demonstrated in other reactions using CML4a as the antigen.

AbBLI (Series G). There are 6 antigens demonstrated in the homologous reaction (labelled a to f). Arc d has a slight diphasic hump and migrated strongly toward the cathode, therefore it is assumed to be gamma globulin. For the most part, the other arcs showed little migration except for c which was on the anode side of the well. Its configuration and band diameter was similar to that usually seen with albumin. All of these arcs are present in the patient's serum except for b. The same reaction occurred with K2a and K2b as with Davis Serum. In CML4a and CML4b, both arcs a and b are missing, but the other 4 are present. It is interesting that K2a and K2b give reactions against this antiserum that are very similar to the reaction AbK2a vs. AgK2a. Davis Plasma (not shown in Fig. 3) was also reacted against this antiserum. The reaction did not differ from that with Davis Serum. J111C

and J111G each reacted with AbBL1 to give one strongly anodic precipitate that was broad and bilaterally diffuse. This antigen could not be identified in AgBL1, even with a special technique that will be discussed later.

Buch. Serum #1 (Series H). This was used in the antibody trough with Buch. Tumor extract in the antigen reservoir. No reaction was noted though the experiment was repeated three times.

Buch. Serum #2 (Series I). This human serum (collected post-operatively) did not react with the patient's own tumor. However, an interesting result did occur against P7a. A broad diffuse arc was present at the point of zero migration and near the antibody trough. This was not present against P7b. Furthermore, no reaction occurred between this serum and K2a, K2b, or Br6.

Gotham Serum (Series J). Using this as antiserum, there was no reaction against P7a or P7b. There were two areas of diffuse precipitation against Buch. Tumor, one anodal and the other cathodal.

Davis Sera #1 and #2 (Series K and L). When the preoperative serum (Davis Serum #1) was reacted against an extract of the patient's own tumor, no reaction occurred (see Row G, Fig.3). However, the same reaction using the patient's post-operative serum demonstrated an arc at the point of zero migration. The implications of this finding will be discussed subsequently.

Gamma Globulin of AbBL1 (Series M). This was reacted against BL1, Davis Serum #1, and Davis Serum #2. In all three instances, only 2 arcs appeared. They corresponded to arcs c and d in row G, Figure 3.

AbBL1a1, 2, 3, and 4 (Series N). Row N contains four antiserum troughs, each filled with one of these antiserums in the same order. They represent absorptions of AbBL1 with different amounts of Davis Serum #1 (Table 1). Each reacted with AgBL1 and Davis Serum #1. None of the reactions demonstrated visible precipitation.

AbBL1a10, 20, and 30. (Series O). Row O represents these three antisera in the same order. The first two antiserum troughs contain AbBL1a10, and so on. These antisera are derived from the absorption of AbBL1 with Davis Serum #2, in different proportions (Table 1). Using AbBL1a10, there was no reaction with AbBL1, Davis Serum #1 or Davis Serum #2. However, with AbBL1a20, arcs b, d, and e are seen in AgBL1 (using code letters from arcs in homologous reaction of AbBL1--Row G). With this same antiserum, arcs b and d are present in Davis Serum #1 and #2. AbBL1a30 gives the same reactions as AbBL1a20, except there is an additional precipitate against AgBL1. It has nearly zero migration and cannot be identified in the homologous reaction in Row G, unless it represents arc a.

AbKC (Series P). The homologous reaction consists of at least 9 sharp, distinct arcs (a to i). Antigen a is the

most strongly cathodic antigen and is diphasic, therefore is considered to be gamma globulin. It is difficult to say with certainty, but f could represent a continuation of a since the two appear to coalesce near the antigen well. If this is true, then the appearance of the two together is similar to that found when macroglobulins are used as antigens. Other antigens migrating toward the cathode include b and c. Arc h is nearest the anode and its configuration and density lead to the conclusion that it is albumin. The other antigens are anodal except for d and e which have migrated little if at all. In the reaction AbKC vs. AgKNC, all antigens that were present in the homologous reaction were seen except for e. In this area, there was a small area of slight diffuse density, but no well defined arc. KG5 and KNG5 gave reactions that were identical to each other except for a similar difference seen in the area of arc e. Arcs a, c, d, g, and h were present in both reactions. In KG5, e was very sharp and distinct, whereas in KNG5 the same area contained a scanty amount of diffuse precipitate. Essentially the same difference existed whenever similar extracts of the kidney tumor and normal kidney were compared using this antiserum.

It seemed of interest to compare Genetron extracts at different stages during the procedure. In the code

employed for their identification, the number following the capital letter "G" (Genetron) designates the number of times Genetron was added, beginning with the distilled water extracts (AgKC and AgKNC). For example, the "G" antigen in Fig. 1 is G5 since the treatment of the Crude antigen with Genetron was repeated for a total of 5 times. As was stated above, the reaction of AbKC with its homologous antigen homogenate contained 9 demonstrable antigens (a to i). After the first Genetron extraction (extracts KG1 and KNG1), the only arc that disappeared was f, though many of those still present were shorter than those of the homologous reaction. It is seen that by the third extraction, antigens i and f have been deleted. There is an arc at the point of zero migration and near the antibody trough that was not previously present in reactions with any of the tissue extracts, but probably corresponds to i in the reaction AbKC vs. York Serum. After 5 extractions, the only arcs present of the original 9 are a, c, d, e, g, and h. Though not shown in Fig. 3, the reactions of AbKC vs. AgKG7 and AgKNG7 were identical with those of AbKG5 vs. AgKG7 and AgKNG7. It is seen that after the seventh extraction with Genetron only 4 antigens could be demonstrated. These are h, d, e, and a or c.

The reaction of AbKC against York Serum presents some important differences from the homologous reaction. Firstly, there is at least one antigen demonstrated that

is not seen in the reaction AbKC vs. AgKC. Arc i is an additional antigen and there are two lines near the cathode end of d. It could not be determined if this represented a split tail on arc d or whether these were two separate antigens. An attempt will be made to explain this finding subsequently.

Arcs corresponding to antigens a, b, c, d, g, and h are present in the reaction with saline extracts KS and KNS. Extract AgSC contains five arcs corresponding to a, d, e, g, and h. The important observation is that e is a sharp, well-defined precipitate. AgANC demonstrates the presence of a, b, g, and h. Using AbKC as the antiserum, no other extracts were shown to contain antigen e. AgSG5 contained a, d, g, h, and possibly c. AgANG5 contained at least two arcs. Arc h was very wide and short and could be hiding arc g, since in the reaction AbKG5 vs. AgANG5 arc g is present as a short line near to, and in line with, arc h. The other precipitate cannot be defined. The reaction of AgANSA is identical to that with AgANC in that arcs a, b, g, and h are present.

AbKG5 (Series Q). Since this antiserum has been prepared using an antigen extract that contains no antigens different from those in AgKC, the arcs in the homologous reaction have been labelled according to the corresponding arcs in the reaction AbKC vs. AgKC. AbKG5 vs. AgKG5 presents 5 well-defined arcs corresponding to a, d, e,

g, and h. The only difference from the reaction with AgKNG5 is due to arc e. As has been stated previously concerning normal kidney extracts, arc e is rather diffuse and ill-defined. For this reason, it has been labelled e' and its significance will be discussed below. The reactions with AgKC and AgKNC were identical to those reactions observed with the corresponding Genetron extracts. AgKG7 and AgKNG7 also gave results identical to those seen using AgKC and AgKNC respectively, except that antigen g has been deleted between the fifth and seventh Genetron extractions. AgKS and AgKNS gave the same results as the homologous reaction, indicating that all of these antigens are soluble in 0.1 M saline. Antigens a, d, and h are present in AgANC and AgSC. AgSC also contains the sharp, well-defined form of e. AgANG5 contains antigens h, g, and one other that cannot be defined. AgSG5, AgSSa and AgANSA are identical to the homologous reaction except that arc g is not present in SG5 and ANSA.

Antigens a, d, g, and h are the only ones demonstrated in York's Serum. This differs from the homologous reaction in that e is not present.

Tumor and normal kidney DNP showed results similar to those when AbKC was used as the antiserum. Each had large, bilaterally diffuse precipitates on the anodal side of the plate and the tumor DNP precipitate appeared 8-12 hours before that of the normal kidney was visible. The

TDNP reaction zone contained an additional diffuse precipitate near the antigen reservoir. Tumor DNA vs. AbKG5 demonstrated an elongated precipitate extending from the antigen well toward the anode. It was very sharply defined and differed in this respect from the non-specific precipitate seen around the well when human serum is used as an antigen. Normal kidney DNA gave an ill-defined area of precipitate similar to the type just described. An important observation would seem to be that both of these antigen preparations precipitated symmetrically relative to the antigen reservoir, even when faced on only one side with the antiserum. The possibility that the precipitate is due to an antigen-antibody reaction is therefore remote.

Cellulose Acetate Electrophoresis

The following extracts were electrophoresed on cellulose acetate strips (Oxoid): AgKC, AgKNC, AgKG5, and AgKNG5. They were subsequently subjected to staining by the Feulgen reaction. All four extracts gave positive reactions on the anodal side of the point of zero migration, indicating the presence of DNA in the solution.

Staining of IEA Plates

In my experience of staining 16 plates (each containing 4 reaction zones) with Buffalo Black, no additional information was elicited. Washing the plates and fixing with acetic acid, as previously described, seems to be sufficient.

No attempt was made to characterize antigens by specific staining.

Temporal Changes During Plate Development

Figure 4 has been presented so the reader can see the changes that occur in a plate as it is developing. This serves to demonstrate why frequent observation and photographic recordings are desirable.

Frozen vs. Refrigerated Reactants

Aliquots of reactants AbKC and AgKC were stored in the freezer at -10°C. and in the refrigerator at 7°C. for four months. They were then reacted against each other in all combinations. There appeared no differences in the number of arcs present, in the distance from the antibody trough that an arc was precipitated, or in the intensity or configuration of a precipitate.

Discussion

Very little use has been made of IEA in the study of human tissue extracts. Some investigators have employed the technique to study human normal and malignant tissues (4,5), but pooled extracts and antisera have been used in these instances. Since isoantigenic differences are present within a species, it would seem that the results of such an investigation could yield no more information than if one were to use different strains of mice and draw conclusions as if one genetically pure strain had been used in the experiment. To my knowledge, the use of IEA in the study of normal and tumor tissue derived from the same organ and the same individual has not been reported prior to this work. Other techniques have been used frequently: cytotoxicity, anaphylaxis, gel diffusion, complement fixation and others. For this reason, few relationships between this work and others can be stated.

In discussing the results, it would seem desirable to follow the sequence employed in the previous section of of the paper. Subsequently, a few statements and conjectures will be made concerning the field of tumor immunology.

Antigen Analysis

Again, the reader is referred to Table 1 and Figure 3 in order to follow what is being stated.

Figure 3 is nearly in chronological order with respect

to the time when the work was performed. It must be conceded immediately that the early antisera are not optimum for the studies carried out. Also, the technical details involved in IEA (as with most other investigative techniques) require repeated practice and experimentation with the particular antigen system before reproducible results can be obtained. As an example, compare the homologous reaction AbK2a vs. AgK2a (5 arcs) with the reaction AbKC vs. AgKC (9 arcs). Not only are there more antigens demonstrated, but the plates were less frequently contaminated, the agar more transparent and the electrophoresis gave better separation in the later work.

AbK2a (Series A). In the homologous reaction of this series, 5 antigens are demonstrated. When the normal tissue extract (AgK2b) was reacted against the same antiserum, only 4 antigens were found; arc e was not seen in the reaction. This brings up the question of whether e represents a tumor-specific antigen. This question becomes more important in view of the fact that this antigen was found in some of the tumor extracts reacted in this series (Br6, K8a and CML4a). The solution to the problem is found by observing the results in series B, where the normal kidney antiserum (AbK2b) was used. The antigen is demonstrated in the following extracts using this antiserum: AgK2a, AgK2b, AgBr6, AgK8a, AgK8b, AgK8c, and AgCML4a. These findings do not rule out the

possibility that this is a specific tumor antigen for the following reason: If it is a tumor antigen and is circulating in the serum (11, 12, 14, 42, 43, and 44), the concentration in the normal kidney extract (AgK2b) might be so small that it cannot be detected immunologically. However, since a concentration as small as 0.1 microgram per cent can elicit an antibody response (15), the finding of an antibody to this antigen in the normal kidney antiserum could be explained by the presence of a tumor antigen that is circulating. If this is truly the explanation however, it would be most difficult to explain the presence of this antigen in AgK8b and AgK8c since they are normal tissue extracts. More information on this point could have been obtained by using the individual's serum as an antigen reactant. This was not possible since the tissues were obtained at necropsy and the value of collecting serum was not foreseen at that time. It would seem that the best explanation for the additional antigen in AgK2a of series A is related to a quantitative difference between the antigen in K2a and the same antigen in K2b. Such quantitative factors probably explain the differences seen in reactions such as AbK2a vs. AgCML4a and AbK2a vs. AgCML4b. Dilution and concentration of reactants was not performed on extracts prior to charging the antigen wells. Such reactions might have demonstrated that quantitative factors were responsible for deletion of some of the arcs

in reactions in which unexplainable results were obtained.

AbK2b (Series B). For the most part, the pertinent findings regarding this antiserum were discussed with Series A. However, important comparison can be made between antisera AbK2b and AbK2a. By observing the reactions in series A (Fig. 3) and comparing these to the corresponding antigen extracts in series B, one can see that AbK2b generally demonstrated more antigens than AbK2a. Differences in arc density (not shown in Fig. 3) also existed indicating differences in antibody titers between the two antisera (13,35). Herein lies one of the disadvantages of comparative immunological investigations--variation of antibody response. Because of this factor, it is important to immunize many animals and to be very selective regarding the antiserum one uses. In situations where it is possible, the most reliable conclusions can be attained by using results obtained with a single antiserum, thereby obviating the difficulties in interpretation of results when using different antisera.

AbP7a (Series C). The homologous reaction with this antiserum produced 3 demonstrable precipitation arcs. An extract (P7b) of a metastatic lesion (found in the liver) of the same tumor showed an additional arc parallel to the anodal arc of the homologous reaction. It precipitated nearer to its point of origin after electrophoresis.

This is an interesting situation since we are dealing with a single antiserum, yet the reaction with AgP7b demonstrates more antigens than the homologous reaction. Therefore, everything else being the same, the explanation must involve a difference in the antigen extracts. In the first place the extra arc could be due to an antigen that cross reacts with an antibody (probably anti-c) and this antigen has a slower diffusion rate or is less concentrated than antigen c. This is doubtful since most precipitates involving cross reacting antigen-antibody complexes appear as diffuse arcs rather than sharp ones. The other explanation involves quantitative factors. Either the antigen is present in large quantities in AgP7a, therefore antigen excess prevents formation of an insoluble complex, or the antigen is in such low concentration in AgP7a that it causes an antibody response, but is not immunologically demonstrable. In any case, there is no evidence that this antigen has any relation to the neoplastic process.

Discrepancies are found between the homologous reaction and those using sera as the antigen. The same explanations are possible with these results. Arcs a and b are present in all three sera and probably represent normal serum antigens. It is interesting that when using the same antiserum against the preoperative and postoperative sera (Buch. Sera #1 and #2) of another tumor patient, the post operative serum demonstrates two more antigens than the former. The

reactions were performed at the same time with the same volume of reactants. It is difficult to see how technique could be involved in explaining this difference. An interesting conjecture would be that the two antigens were released into the circulation during the surgical procedure and antibodies to these same antigens were contained in AbP7a. The extra cathodal antigen is also present in Gotham Serum.

AbP7b (Series D). This antiserum generally forms more arcs against the different extracts than AbP7a. The immunizing extract must be assumed to have contained normal liver components. Arcs b, c, d, and e are shared by AgCML4a and 4b and probably represent serum antigens and/or liver antigens. Arcs b, c, and e are almost certainly circulating antigens since they are also found in Gotham Serum and Buch. Serum #2. It is not known why Buch. Serum #1 was not reacted with this antiserum.

AbBr6 (Series E). Arcs c, d, and probably a are present in Gotham Serum, Buch. Serum #1 and Buch. Serum #2. Using this antiserum, no difference is seen between Buch. Serum #1 and #2. Note also that in Series C neither of the extra arcs in Buch. Serum #2 are seen in AgBr6. It is doubtful that they are present in the extract AgBr6. Antigens b, c, and d most likely represent normal serum antigens.

<u>AbCML4a (Series F).</u> In the reaction with Buch. Sera #1 and #2, the postoperative serum again contains an extra arc. It does not correspond to either of the two previously discussed. Again, the reactions were performed at the same time and under similar conditions. The only explanation would seem to be that an antigen is liberated into the serum during the surgical procedure or there are quantitative differences between the preoperative and postoperative sera. Gotham Serum also contains this extra antigen. The possibility exists that these unique arcs represent circulating tumor antigens, but evidence supporting this is lacking. As previously stated, technique, experimental design, and antisera reactants were not of desirable quality in these early experiments.

<u>AbBL1 (Series G).</u> Extract AgBL1 contains a minimum of 6 antigens, 5 of which are demonstrated in the patient's serum (Davis Serum). The kidney tumor and normal kidney extracts (K2a and K2b) gave reactions identical to that with Davis Serum. It is possible that antigen <u>a</u> is a tumor antigen that is also present in the serum of the donor of AgK2a and AgK2b. It is also possible that arcs <u>a</u>, <u>c</u>, <u>d</u>, <u>e</u>, and <u>f</u> represent normal serum antigens. Antigen <u>a</u> might also be a normal tissue antigen shared by kidney and bladder. This would explain why it is not found in AgCML4a and 4b. Necrotic areas in the tumor could explain its release into the serum. If this is true though, one

would expect to have demonstrated arc b in the serum also. It is demonstrated only in the homologous reaction. Regarding this, it would seem desirable to discuss Series K and L at this time. The antisera used in these two reactions were the preoperative and postoperative sera respectively from the donor of BL1. His preoperative serum did not reveal a reaction against the saline extract of his own tumor, whereas his postoperative serum presented an arc in a similar position as arc b in the homologous reaction. This finding is similar to that of Graham and Graham (23). They suggested that it might represent the *in vivo* absorption of antibody by the patient's own tumor, with a detectable titer available after removal of some of the tumor mass. It is also interesting that this arc is situated in a similar position as arc e in Series P and Q which will be discussed subsequently.

Extracts J111C and J111G reacted with AbBL1 to give a diffuse anodal arc. A corresponding antigen could not be demonstrated in AgBL1, even with the following special technique. The center antigen reservoir was filled with J111C or J111G. After electrophoresis, one of the antibody troughs was filled with AbBL1 and the other was filled with AgBL1. The precipitation occurs in the zone between J111 and AbBL1. Since AgBL1 is diffusing in a straight line toward AbBL1, any precipitation occurring between the two will be in the form of a straight line.

Since J111 is diffusing from a point source, it will diffuse radially and precipitation will occur in the form of an arc. If the antigen in question is contained in AgBL1, the ends of the arc will continue as straight lines parallel to the antibody troughs. This did not occur, therefore the antigen could not be demonstrated to be present in AgBL1. The diffuseness of the arc would suggest that the antigen was cross reacting with the antibody (13).

Buchner Sera #1 and #2 (Series H and I). Buchner Serum #1 was reacted with an extract of the patient's tumor. No demonstrable reaction occurred, though the experiment was repeated three times. The same was true for Buch. Serum #2 vs. Buch. Tumor. Buch Serum #2 reacted with AgP7a to give a broad, diffuse arc at the point of zero migration and near the antibody trough. No such reaction occurred with P7b, Br6, K2a, or K2b. Since the arc is near the antibody trough, the antigen must be in high concentration and/or must be rapidly diffusing. The density of the precipitate favors the latter (35). Since the two reactants were from different individuals the possibility of an isoimmune reaction exists. Since P7b is from the same patient as P7a, this explanation is unlikely. The shape of the precipitate makes it unlikely that the arc is an artifact. If one rules out an isoimmune reaction and an artifact, it would seem that the only explanation for the absence of a reaction with Buch. Tumor would be on a

quantitative basis; possibly antigen excess preventing the formation of an insoluble complex.

Gotham Serum (Series J). This antiserum did not react with $Ag^{P}7a$ or $Ag^{P}7b$. Two arcs, one anodal and one cathodal, appeared in the reaction with Buch. Tumor. It is interesting that those two arcs have similar electrophoretic mobilities similar to those discussed previously in Buch. Serum vs. $Ab^{P}7a$ (Series C). It is possible that this represents the reaction of a naturally occurring isoantibody. Another suggestion is that the arcs represent the reaction of antibodies formed by Gotham against his own tumor with similar tumor antigens in Buch. Tumor. If this is so, the broad, diffuse arc suggests the reaction is between antiserum prepared against one antigen with a heterologous but crossreacting antigen (13). Indirect evidence for this exists in the following observations: Three demonstrable reactions have occurred using human patient's serum as the antibody source (Series I, J, and L). Two reactions were between one patient's serum and the extract of another patient's tumor. These formed broad, diffuse arcs. The other reaction (Series L) was with a patient's serum and an extract of his own tumor. This arc was sharp, indicating a more specific antibody for this antigen (as one would expect, since it was the antigen that elicited the antibody response and is therefore homologous).

Gamma globulin of AbBL1 (Series M). This antiserum source reacted with AgBL1, Davis Serum #1, and Davis Serum #2 and demonstrated arcs corresponding to c and d in Series G. The yield of gamma globulin was low as indicated by paper electrophoresis and subsequent scanning. This is considered to be due to faulty technique. Since no advantage could be foreseen in fractionating antisera, further attempts in this direction were not made.

AbBL1a1, 2, 3, and 4 (Series N). Antiserum AbBL1 was absorbed with Davis Serum #1 (Table 1). No reaction occurred when these antisera were reacted with AgBL1 and Davis Serum #1. This would seem to indicate that all antigens in the homologous reaction of Series G are contained in Davis Serum #1, and that all antibodies for these have been absorbed from AbBL1. This may well be the case. Also, the amount of Davis Serum added in the absorption may have produced an antigen excess such that when the reactions were performed insoluble complexes could not be formed.

AbBL1a10, 20, and 30 (Series O). These antisera are similar to those in the previous series, except that Davis Serum #2 was used for the absorption and different proportions of reactants were used. AbBL1a10 did not demonstrate a reaction with any of the antigen reactants. Note that the proportion used in the absorption is similar to that in AbBL1a1, which gave a similar result. AbBL1a20

was absorbed with a greater dilution than any of the preceding antisera. In its reaction with AgBL1, observe that arcs b, d, and e of Series G are present. Davis Sera #1 and #2 show arcs b and d. AbBL1a30 gave similar results except for an additional arc against AgBL1. This may be arc a, which is more concentrated relative to its antibody than in the homologous reaction of Series G. It should be stated that absorptions performed with multiple antigens in the system should be considered less than adequate, since different proportions exist in each antigen-antibody system and different equivalence zones are present. Serial dilutions of the antigen reactants should have been performed, and each used in the antigen reservoir. It is probably the case that the antibodies that were still demonstrably active were not absorbed out of the antisera because of the high dilution, or the antigen excess factor was not strong enough to prevent precipitation. No difference is seen between Davis Sera #1 and #2 in these reactions. The 3 arcs seen in AbBL1a20 vs. AgBL1 are seen in Series G (Davis Serum), therefore they are circulating antigens. This is not true for the additional arc in AbBL1a30 vs. AgBL1; therefore it may represent at least a tissue antigen if not a tumor antigen. Note that it is in the same position as the arc in Series L.

AbKC(Series P). The homologous reaction demonstrates the presence of at least 9 antigens in AgKC. At least one

other antigen is demonstrated in York Serum. This is easily explained by the presence of serum in the immunizing extracts. Since the amount of antigen necessary to elicit an antibody response is very small, an antibody was formed against it. The low concentration of the antigen in the extract could preclude its demonstration in the homologous reaction.

The finding of most significance is that of a sharp, distinct arc g in the extract of the kidney tumor (KC), but not in that of the normal kidney (KNC). It was also noted to be present in AgSC of this series. Further discussion of this finding will be presented subsequently.

Antigens a, b, c, d, g, and h are shown to be soluble in 0.1 M saline by the reactions of AgKS and AgKNS vs. AbKC.

An interesting result occurred when tumor DNP (TDNP) and normal kidney DNP (NDNP) were compared by their reactions with this antiserum. The broad anodal precipitate in TDNP was seen 8-12 hours before its counterpart in NDNP. Since the precipitate in TDNP is situated nearer the antibody reservoir, it seems likely that the time differential could be explained by a situation where the concentration in NDNP was significantly smaller, therefore requiring longer time for its concentration to build up sufficiently to cause the visible precipitation of the antigen-antibody complex. It is interesting that the location of this arc

is similar to that found in J111C and J111G in Series G. In the TDNP reaction, there was an additional area of very light diffuse precipitate (stippled area of Fig. 3) near the antigen reservoir. Its significance is not known, though it does represent a difference from NDNP. This could be explained on a quantitative rather than qualitative basis. However, Lomakin (39) found that tumor DNP possessed some degree of specificity, but contained the same antigens as DNP from normal tissue. This could explain the extra zone of precipitate in TDNP. It is likely that precipitin antibodies are formed against DNP because the immunological activity in horse serum to DNP is in the gamma globulin fraction (26). The presence of the other partial arcs in these reactions are indicative of antigens still present after the DNP extractions. It has been assumed that the broad arcs represent DNP for the following reasons: (1) the spectrophotometric scans of the extracts gave typical results for the presence of DNA and DNP, with a peak absorption at 260 millimicrons and a minimum at 237 millimicrons, (2) the extracts used for immunization gave positive Feulgen reactions, and (3) the arcs were deleted in the DNA extracts made from the DNP solution.

The reactions of this antiserum with extracts at different stages in the Genetron extraction have been described and do not require further discussion.

<u>AbKG5 (Series Q).</u> The finding that the homologous reaction and that with AgKG give identical results, would seem to indicate that all of the other antigens found in the homologous reaction of Series P have been removed by the Genetron extraction, since remaining small quantities would probably have stimulated an antibody response.

As stated previously, the most significant finding concerns arc <u>e</u>. In the homologous reaction, it is found to be very sharp and prominent. In the reaction of AbKG5 vs. AgKNG5, it is consistently diffuse with a suggestion of having a slightly sharper edge on the side of the antigen reactant. Because of this difference, it has been labelled <u>e'</u>, to emphasize this difference. The following situations could explain this finding: (1) antigen excess--if this is the cause, the side nearer the antiserum trough would be the sharpest and the arc would migrate toward the antibody trough. These events did not occur. Nor did the arc become sharper when AgKNG5 was diluted one-half. (2) relative antibody excess--the arc would have been sharp during its early appearance and it would have become broad and very dense. This did not occur.

If the band can be described as bilaterally diffuse (this seems to be the case), it suggests that the antigen is not entirely homogeneous in combining ability with available antibody. It has been shown that such an arc can be caused by the reaction between antiserum prepared against

one antigen with a heterologous but cross-reacting antigen (13). Witebsky, et al.(63) have suggested that one of the difficulties of demonstrating tumor antigens by complement fixation may be that they are closely related to normal tissue antigens.

McKenna, et al. (48) found that antigens detectable by complement fixation remained in tumor extracts, but did not remain in normal tissue extracts. Their Genetron extracts of normal tissues contained no protein precipitable by trichloroacetic acid. Contrary to these findings, in the present studies, both normal kidney and kidney tumor contained precipitable material after 7 Genetron extractions. Also, with IEA, a minimum of 5 antigens were demonstrated in KG5 and KNG5, and 4 antigens were demonstrable in KG7 and KNG7. DeCarvalho (15) has suggested that the extraction procedure may have to be repeated as many as 16 times. He found that only one band remained after Genetron extraction of human leukemic cells, followed by absorption with rabbit anti-human amnion serum. This was interpreted to be a specific leukemic antigen. Such a conclusion cannot be made, since it assumes that human amnion contains all normal antigens that are present in the human. It would seem desirable to compare tumors exclusively with the normal tissue of origin collected from the same individual.

All antigens demonstrable in the homologous reaction are also present in AgKS, indicating that they are all

soluble in 0.1 M saline. Antigen e is found in all extracts of the seminoma (SC, SG5, and SSA) and in the saline extract (ANSA) of a bladder tumor. It is uniformly absent from York Serum. This finding is compatible with those of other investigators (48) who report the presence of the antigen in patient's serum only 25 per cent of the time.

This antiserum gave results similar to those of AbKC when reactedwith the DNP extracts, except that the arcs due to probable contaminants have been deleted.

The results in TDNA and NDNA have been stated with a word about their significance in the previous section of this paper. Because these precipitates appear to be non-specific, it is interesting to note that some substances that are soluble in agar gel while attached to another molecule (eg. DNA-protein), become insoluble when separated (13).

There is evidence that the density near the antigen reservoir in reactions where serum is used as the antigen reactant represents the precipitation of beta-lipoprotein in the agar, due to poor solubility and an actual interaction with the agar (13).

Taylor, et al. (57) state that Genetron has been used to remove nonviral protein from virus-tissue extracts. They suggest that, "Such treatment may be a means of isolating viruses or viruslike material from tumor tissues in which a virus etiology has not yet been established." The Genetron extraction of the rhesus monkey histiocytoma

(virus-caused) was said to produce a virus suspension with fewer tissue proteins (49). It is difficult to say how these findings relate to the work in this paper, since no absolute difference was found between the normal and malignant kidney tissue, though Genetron was shown to remove some normal antigens. The difference seemed to be that of cross-reacting antigens (arcs e and e').

Because of the varied findings in the literature concerning specific tumor antigens, (loss of antigens, gain of antigens and no demonstrable change), an interesting conjecture can be made. It would seem likely that observations of antigenic differences in tumor and normal tissue merely reflect secondary changes in the cell. This may indicate that the basic defect is often related to protein synthesis, therefore denoting a change in DNA, RNA, or the endoplasmic reticulum. This is in agreement with many of the investigations previously cited (3, 20, 52, 55, 59) and with the fact that many animal tumors have viral etiologies. It would seem then that the many known causes of carcinogenesis, such as radiation, mutation, viruses, chemicals and other physical or chemical agents, act by altering the previously stated cell components. Whether the basic defect must result in change of a specific gene locus or the reactions controlled by it, or whether many loci can be involved individually or in multiples (eg. Osgood (52) to cause neoplastic growth, we can only surmise.

In the investigations in which Genetron extracts have been shown to demonstrate specific antigens in tumors (15, 48, 49, 57), it appears to this writer that the findings could be interpreted as being due either to viral proteins or to changes in tissue proteins. If some human tumors have a viral etiology and if viral antigens are being demonstrated, then this could account for the disparity of findings by different investigators. Greene (25) has cited evidence that the virus of the Shope Papilloma cannot be isolated from the tissue at certain stages of development of the tumor, yet the Rous sarcoma contains virus in large quantities at all times. The work by Bendich (3), in which the carcinogenic activity of the Polyoma virus was found to be in the DNA, implies that secondary changes in protein synthesis could occur in the cell by the interference of virus DNA with the cellular DNA.

In conclusion, the following situations would explain antigenic differences between normal and malignant tissues: (1) the tumor synthesizes an antigen that is not present in normal tissue, (2) the tumor fails to produce one of the normal antigens, (3) the tumor synthesizes a protein similar to a normal antigen, and (4) an antigen is present that does not have its origin in the host (eg. virus protein). Of course, combinations of these might occur. Only the latter contains an implication of cause and effect.

If antigenic differences exist, of what importance are they? It would seem that the principle significance of such differences may lie in their value in diagnosis and/or therapy of neoplastic disease. Understanding the basic mechanisms of malignant growth will almost certainly require knowledge of the fundamental processes of normal differentiation and development.

Temporal Changes During Plate Development (Fig. 4)

Figure 4 emphasizes the necessity of frequent recording of results during plate development. It does not demonstrate the disappearance of any arcs, though this does occasionally occur. The broad anodal and cathodal arcs are albumin and gamma globulin respectively. Their density indicates large antibody concentrations, as does their progressive increase in breadth. This is the reason that high titers are sometimes detrimental in this technique since the increase in breadth can cover other arcs in close proximity to these. It also demonstrates one of the advantages of R-type antiserum over H-type antiserum. In such antibody excess, using H-type antiserum, the antigen-antibody complex would become soluble and precipitation would be prevented. In Fig. 4f there are many examples of secondary (Liesegang) precipitation due to excess reactant. In this instance, the strong antibody at first resulted in a primary precipitation (heavy band near antibody trough), then because antigen could not diffuse fast enough to react

with great excess of antibody that was present in this area, precipitation did not occur. Consequently, immediately beyond the primary precipitate, there is a clear area where antibody excess prevented precipitation, followed by a broad area of poor precipitation which occurred under the disadvantage of antibody excess. Finally another heavy precipitate occurs where the diffusion distance for antibody has tended to neutralize its initial overwhelming advantage over antigen. The ends of the primary and secondary precipitates are seen to join. It can be determined from this figure that antibody is of R- rather than H-type since the primary precipitate resisted dissolution in the face of excess antibody (13). As would be expected, this phenomenon is related to time as well as reactant concentration.

Disadvantages of the Immunological Approach to this Problem

A minor complication in immunodiffusion tests can produce results that can mislead and severely handicap the investigator to the degree that trends in his work will be altered by the aberrations in his results, or even to the degree that conclusions will be made that are not appropriate. Often, simple ancillary procedures will more clearly define the situation; for instance, concentration and dilution of reactants should be performed. For these reasons, some of the earlier results reported here are not considered to represent optimum yield of information.

Other problems include: (1) non-specific reaction of reactants with the gel, (2) reactant stability during the time one is waiting for antisera to be formed, (3) the fact that this technique detects only precipitin reactions, (4) individual variation in immune responses, and (5) relatively low antigenicity of some of the tissue substances.

It would seem that the most significant mistake made in the work reported here is the attempt to study too many tumors and the lack of more careful selection of tissues. The most reliable results can be obtained when both tumor and normal tissue have been collected at surgery from the same individual, followed immediately by extraction and storage of materials. Then, with careful avoidance of the aforementioned difficulties, and the use of appropriate ancillary techniques, one should be able to obtain informative, reliable, and easily reproducible data.

Possibilities for Future Related Investigations

If specific tumor antigens are found, attempts should be made to isolate them and study their physicochemical properties and biological activities. If obtained in pure form, antiserum should be prepared and tagged in such a way that the localization of the site of the reaction in the cell could be demonstrated *in vivo* or *in vitro*.

If tumor-specific antigens are contained in the serum of some patients, it may be possible to establish serological methods of diagnosis and even screening methods

applicable to large populations.

It is also conceivable that immune therapy (active or passive) of malignant disease may someday be possible. The isolation of tumor-specific antigens would greatly enhance this possibility. The fluorocarbon extraction method employed in the work herein reported might be a step in this direction and merits further investigation.

Most certainly, studies concerning nucleic acids and nucleoproteins promise to reveal considerable information concerning the processes that occur in both the normal and abnormal cell.

SUMMARY

Immunoelectrophoretic analysis has been performed on human normal and malignant tissues. An antigenic difference has been found between extracts of normal kidney and kidney tumor obtained from the same individual. This difference appears to be slight; the antigen in the normal tissue appearing to cross-react with the tumor antiserum. It could not be demonstrated in the patient's serum.

Some of the sera obtained from patients with tumors were seen to react with extracts of various tumors.

A fluorocarbon extraction procedure has been tried and found to be useful in removing some of the normal tissue antigens from tumor tissue homogenates.

Investigations relating to tumor-specific antigens have been discussed and certain conjectures regarding carcinogenesis have been made.

REFERENCES

1. Baruah, B.D. Cellular reactions following tumor growth with special reference to plasma cellular response. Cancer Res., 1960. 20, 1184-1194.

2. Batchelor, J.R. Some immunological aspects of cancer. Med. Press, 1961. 245, 346-349.

3. Bendich, A. Nucleic acids and the genesis of cancer. Bull. N.Y. Acad. Med., 1961. 37, 661-674.

4. Bjorklund, B. Antigenicity of malignant and normal human tissues by gel diffusion techniques. Int. Arch. Allergy, 1956. 8, 179-192.

5. Bjorklund, B., Bjorklund V., & Hedlof, I. Antigenicity of pooled human malignant and normal tissues by cytoimmunological technique. J. Nat. Cancer Inst., 1961. 26, 533-555.

6. Black, M.M., Kerpe, S., & Speer, F.D. Lymph node structure in patients with cancer of the breast. Amer. J. Path., 1953. 24, 505.

7. Black, M.M., Opler, S.R., & Speer, F.D. Microscopic structure of gastric carcinomas and their regional lymph nodes in relation to survival. Surg. Gynec. Obstet., 1954. 98, 725.

8. Black, M.M., Speer, F.D., & Opler, S.R. Some components of biologic predeterminism in cancer. Surg. Gynec. Obstet., Internat. Obstr. Surg., 1956. 102, 223.

9. Black, M.M., Opler, S.R., & Speer, F.D. Structural representations of tumor host relationships in gastric carcinoma. Surg. Gynec. Obstet., 1956. 102, 599.

10. Black, M.M., & Speer, F.D. Immunology of cancer. Surg. Gynec. Obstet., Internat. Obstr. Surg., 1959. 109, 105.

11. Burrows, D. Schultz-Dale test for detection of specific antigen in sera of patients with carcinoma. Brit. Med. J., 1958. 1, 368-370.

12. Burrows, D., & Neill, D.W. Investigation of the immunologically active constituent of serum of patients with carcinoma. Brit. Med. J., 1958. 1, 370-371.

13. Crowle, A.J. (Ed.) Immunodiffusion. New York, N.Y.: Academic Press, 1961.

14. Darcy, D.A. Immunological discrimination between the blood of normal and tumor-bearing rats. Nature (Lond.), 1955. 176, 643-644.

15. DeCarvalho, S. Segregation of antigens from human leukemic and tumoral cells by fluorocarbon extraction. J. Lab. Clin. Med., 1960. 56, 333-341.

16. Everson, T.C., & Cole, W.H. Spontaneous regression of malignant disease. J.A.M.A., 1959. 169, 1758.

17. Feldman, M. Immunological components of carcinogenesis. Acta Un. Int. Cancr., 1961. 17, 234-243.

18. Freund, J. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Amer. J. Clin. Path., 1951. 21, 645-656.

19. Freund, J., Thomson, K.J., Hough, H.B., Sommer, H.E., & Pisani, T.M. Antibody formation and sensitization with the aid of adjuvants. J. Immun., 1948. 60, 383-398.

20. Goldthwait, D.A. Nucleic acids and cancer. Amer. J. Med., 1960. 29, 1034-1059.

21. Grabar, P. Agar gel diffusion and electrophoresis. Ann. N.Y. Acad. Sci., 1957. 69, 591-607.

22. Grabar, P. Immunoelectrophoretic analysis. In D. Glick (Ed.) Methods of biochemical analysis. New York, N.Y.: Interscience Publishers, Inc., 1959. pp. 1-39.

23. Graham, J.B., & Graham, R.M. Antibodies elicited by cancer in patients. Cancer, 1955. 8, 409.

24. Green, H.N. The immunologic theory of cancer. J. Chron. Dis., 1958. 8, 123-135.

25. Greene, H.S.N. A virus etiology of cancer? In N. Kaliss (Ed.) Symposium on normal and abnormal differentiation and development. Nat. Cancer Inst. Monogr., 1960, No. 2.

26. Grigor'yan, D.G. The production of specific antisera to the nucleoproteins of human carcinoma tissues. Bull. Exptl. Biol. Med., 1959. 47, 231-235

27. Hauschka, T.S. Immunologic aspects of cancer: A Review. Cancer Res., 1952. 12, 615-629.

28. Heidelberger, M., Turner, J.C., & Soo Hoo, C.M. Preparation of rabbit globulin for therapeutic use by precipitation with sodium sulfate. Proc. Soc. Exp. Biol. Med., 1938. 37, 734.

29. Hirsch, H.M., Bittner, J.J., Cole, H., & Iversen, I. Can the inbred mouse be immunised against its own tumor? Cancer Res., 1958. 18, 344.

30. Hirschfeld, J. Immunoelectrophoresis--Procedure and application to the study of group-specific variations in sera. Sci. Tools, 1960. 7, 18-25.

31. Hunter, J.R. Photographic recording of agar diffusion plates. Nature (Lond.), 1959. 183, 1283-1284.

32. Kabat, E.A., & Mayer, M.M. (Eds.) Experimental Immunochemistry. Springfield, Ill.: Charles C. Thomas, 1961.

33. Kidd, J.G. Distinctive constituents of tumor cells and their possible relations to the phenomena of autonomy, anaplasia, and cancer causation. Cold Spring Harbor Symposia Quant. Biol., 1946. 2, 94-110.

34. Kohn, J. A simple method for the concentration of fluids containing protein. Nature (Lond.), 1959. 183, 1055.

35. Korngold, L. The distribution and immunochemical properties of human tissue and tumor antigens. Ann. N.Y. Acad. Sci., 1957. 69, 681-697.

36. Korngold, L., & Van Leeuwen, G. Immunological and electrophoretic studies of human tissue and tumor antigens. Cancer Res., 1957. 17, 775-779.

37. Korngold, L., Van Leeuwen, G., & Engle, R.L., Jr. Diagnosis of multiple myeloma and macroglobulinemia by the Ouchterlony gel diffusion technique. Ann. N.Y. Acad. Sci., 1962. 101, 203-220.

38. Kosyakov, P.N., & Korostelova, V.S. Carcinomas with identical and with different specific antigens. Bull. Exptl. Biol. Med., 1959. 47, 226-230.

39. Lomakin, M.S., & Malina, Yu. F. The comparative study of the antigenic structure of tumor tissue and homologous normal tissue. Bull. Exptl. Biol. Med., 1961. 51, 349-354.

40. Lovell, E.S. Unusual natural immunity to cancer. J.A.M.A., 1960. 174, 310.

41. Lyons R.B. Antigenic anatomy of the sea urchin--Strongylocentrotus purpuratus. Unpublished master's dissertation, Univ. of Oregon Med. School, 1960.

42. Makari, J.G. Detection of antigens in sera of patients with neoplastic disease by Schultz-Dale test: Its possible use as a screening procedure for tumors. Brit. Med. J., 1958. 2, 358-361.

43. Makari, J.G. The polysaccharide behavior of cancer antigens. Brit. Med. J., 1958. 2, 355-358.

44. Makari, J.G., & Huck, M.C. Use of Schultz-Dale test for detection of specific antigens in sera of patients with cancer. Brit. Med. J., 1955. 2, 1291-1295.

45. Malinin, T.I. Protein in malignant tissue cell. Cancer, 1960. 13, 871-877.

46. Mann, L.S., & Welker, W.H. A specific antiserum for cancer protein. Amer. J. Cancer, 1940. 39, 360-364.

47. Mann, L.S., & Welker, W.H. Further studies of specific precipitin antiserums for the protein of cancer tissue. Cancer Res., 1943. 3, 193-195.

48. McKenna, J.M., Sanderson, R.P., & Blakemore, W.S. Extraction of distinctive antigens from neoplastic tissue. Science, 1962. 135, 370-371.

49. Metzgar, R.S., Grace, J.T., Jr., & Sproul, E.E. Immunologic studies of subcutaneous virus-induced histiocytomas in primates. Ann. N.Y. Acad. Sci., 1962. 101, 192-202.

50. Milgrom, F. A short review of immunological investigations on cancer. Cancer Res., 1961. 21, 862-868.

51. Nairn, R.C., Richmond, H.G., & McEntegart, M.G. Immunological differences between normal and malignant cells. Brit. Med. J., 1960. 2, 1335.

52. Osgood, E.E. A unifying concept of the etiology of the leukemias, lymphomas, and cancers. J. Nat. Cancer Inst., 1957. 18, 155.

53. Ouchterlony, O. Diffusion in gel methods for immunologic analysis. Progr. Allergy, 1958. 5, 1-78.

54. Shope, R.E. Koch's postulates and a viral cause of human cancer. Cancer Res., 1960. 20, 1119-1120.

55. Southam, C.M. Viruses in the field of cancer. Mod. Med., 1960, 28, 74.

56. Southam, C.M., Moore, A.E., & Rhoads, C.P. Homotransplantation of human cell lines. Science, 1957. 125, 158.

57. Taylor, A.R., Gillen, A., & Brandon, F.B. Complement fixing antigens in neoplastic tissue extracts. Virology, 1959. 7, 348-351.

58. Toolan, H.W., & Wallace, R.A. The relative ability of cell-free fractions of the transplantable human tumor, H. Ep. #3, to produce H.Ep. #3 cytotoxins in rats. Cancer Res., 1958. 18, 698-705.

59. Wakonig-Vaartaja, R. Search for the essential factors of carcinogenesis. Ann. N.Y. Acad. Sci., 1963. 105, 1-24.

60. Weiler, E. Loss of specific cell antigen in relation to carcinogenesis. In G.E.W. Wolstenholme and M. O'Connor (Eds.) Carcinogenesis. Boston: Little, Brown, 1959. pp. 165-175.

61. Wigzell, H. Immunological depression of tumor growth in F1 hybrid/parental strain systems. Cancer Res., 1961. 21, 365-370.

62. Williams, C.A., Jr. Immunoelectrophoresis. Sci. Amer., 1960. 202, 130-140.

63. Witebsky, E., Rose, N.R., & Shulman, S. Studies of normal and malignant tissue antigens. Cancer Res., 1956. 16, 831.

64. Wodehouse, R.P. Gel diffusion: A quasi-critical review of recent literature. Ann. Allergy, 1956. 14, 96-113.

65. Zilber, L.A. Specific tumor antigens. Advance Cancer Res., 1958. 5, 291-329.

66. Zilber, L.A. A study of tumour antigens. Acta. Un. Int. Cancr., 1959. 15, 933-939.

Table 1

Tabular summary of reactants. The name of the patient indicates those reactants from the same individual.

Code	Extraction	Dialysis	Tissue	Source	Patient
AgK2a	0.1 M NaCl	yes	Renal Ca	Surg.	Smith
AgK2b	"	"	Normal Kidney	"	"
AgK2c	(Macroglobulin)				"
AgPAR3	0.1 M NaCl	yes	Parotid Tumor	Surg.	Jones
AgCML4a	"	"	Colon Ca in Liver	Autopsy	Daley
AgCML4b	"	"	Normal Liver	"	"
AgBr6	"	"	Ca Lung	"	Jenson
AgP7a	"	"	Panc. Ca	"	Hall
AgP7b	"	"	Panc. Ca in Liver	"	"
AgK8a	"	"	Renal Ca in Lung	"	James
AgK8b	"	"	Normal Kidney	"	"
AgK8c	"	"	Normal Lung	"	"
Buch. Tumor	"	"	Fibrosarc.	Surg.	Buch.
AgBL1	"	"	Bladder Ca	"	Davis
J111C	D. Water	No	Leukemia	Tissue Cult.	
J111G	Genetron	"	"	"	"
AgSC	D. Water	"	Seminoma	Surg.	Clark
AgSG5	Genetron	"	"	"	"
AgSSa	0.1 M NaCl	"	"	"	"
AgANC	D. Water	"	Bladder Ca	"	Aneg.
AgANG5	Genetron	"	"	"	"
AgANSa	0.1 M NaCl	"	"	"	"

Code	Extraction	Dialysis	Tissue	Source	Patient
AgKC	D. Water	No	Renal Ca	Surg.	York
AgKG5	Genetron	"	"	"	"
AgKG1	"	"	"	"	"
AgKG3	"	"	"	"	"
AgKG7	"	"	"	"	"
AgKS	0.1 M NaCl	"	"	"	"
AgKNC	D. Water	"	Normal Kidney	"	"
AgKNG5	Genetron	"	"	"	"
AgKNG1	"	"	"	"	"
AgKNG3	"	"	"	"	"
AgKNG7	"	"	"	"	"
AgKNS	0.1 M NaCl	"	"	"	"
TDNP	DNP	"	Renal Ca	"	"
NDNP	"	"	Normal Kidney	"	"
York Serum					"
Gotham Serum					Gotham
Buch. Serum #1 (pre-operative)					Buch.
Buch. Serum #2 (post-operative)					Buch.
Davis Serum #1 (preoperative)					Davis
Davis Serum #2 (post-operative)					Davis

Code						
AbBL1a1	1 vol.	Davis	Serum	#1	: 10 vol.	AbBL1
AbBL1a2	1 vol.	"	"	"	: 20 vol.	"
AbBL1a3	1 vol.	"	"	"	: 40 vol.	"
AbBL1a4	1 vol.	"	"	"	: 2 vol.	"

Code	Extraction	Dialysis	Tissue	Source	Patient
AbBL1a10	1 vol. Davis Serum #2 : 10 vol. AbBL1				
AbBL1a20	1 vol. " " " : 50 vol. "				
AbBL1a30	1 vol. " " " : 2500 vol. "				

Figure 1

Flow diagram of extraction procedures used in this work.

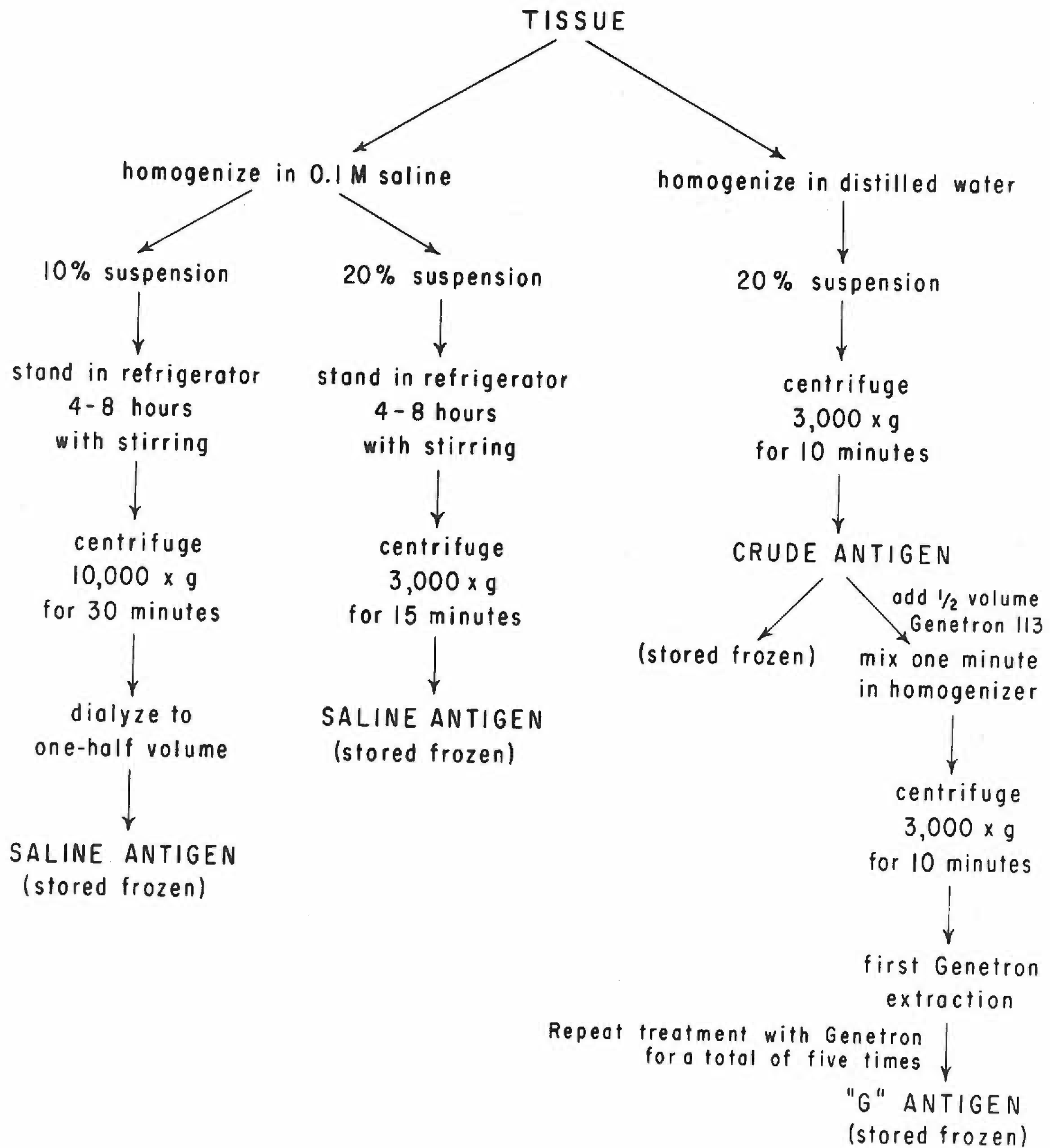
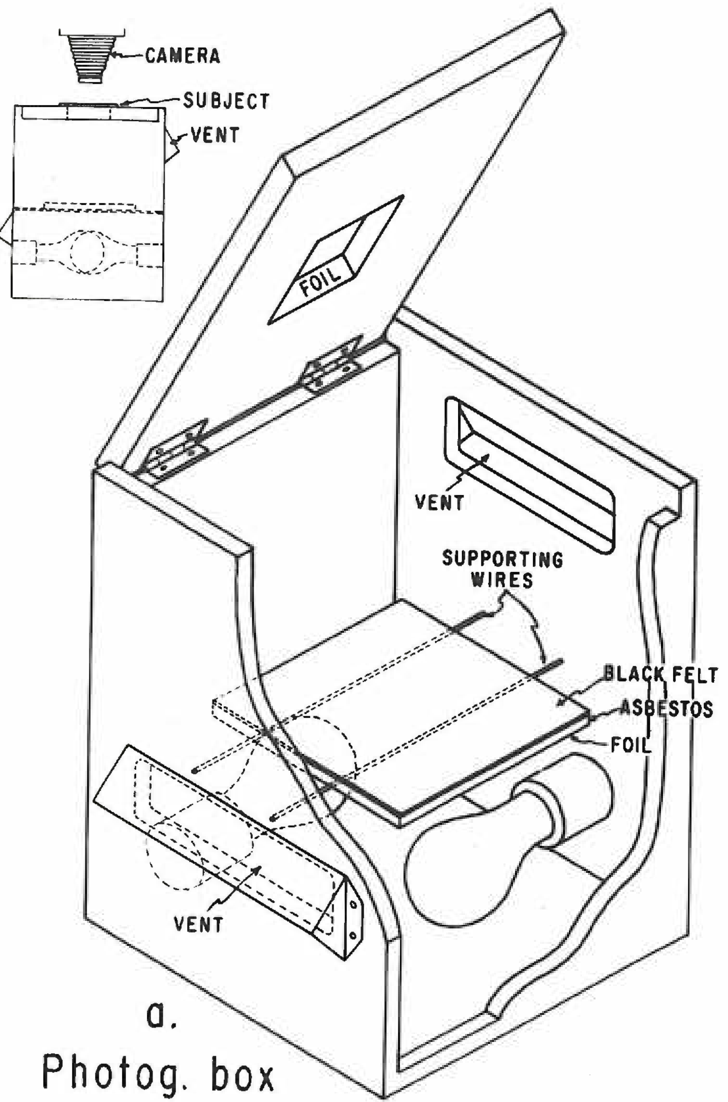
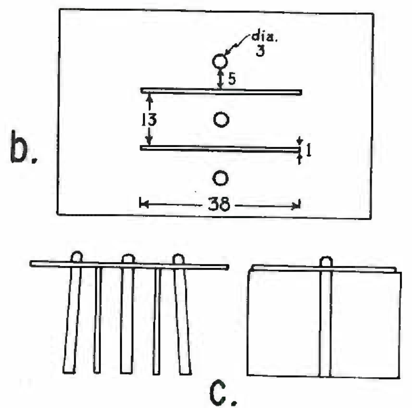
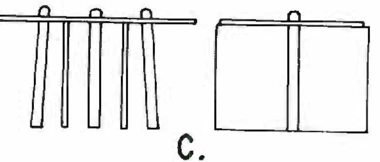

TISSUE
homogenize in 0.1 M saline
homogenize in distilled water
10% suspension
20% suspension
20% suspension
stand in refrigerator
4-8 hours
with stirring
stand in refrigerator
4-8 hours
with stirring
centrifuge
3,000 x g
for 10 minutes
centrifuge
10,000 x g
for 30 minutes
centrifuge
3,000 x g
for 15 minutes
CRUDE ANTIGEN
add 1/2 volume
Genetron 113
(stored frozen)
mix one minute
in homogenizer
dialyze to
one-half volume
SALINE ANTIGEN
(stored frozen)
centrifuge
3,000 x g
for 10 minutes
SALINE ANTIGEN
(stored frozen)
first Genetron
extraction
Repeat treatment with Genetron
for a total of five times
"G" ANTIGEN
(stored frozen)

Figure 2

Equipment used in IEA. (a) Box designed for visualizing and photographing plates. This provides oblique light and a dark background. (b) View of agar plate from above with dimensions in millimeters. (c) Templates used in forming reservoirs in the agar; viewed from adjacent sides. (d) Two agar plates are shown in place in a Durrum cell (cover not included).


CAMERA
SUBJECT
VENT
FOIL
VENT
SUPPORTING WIRES
BLACK FELT
ASBESTOS
FOIL
VENT
a.
Photog. box


b.
dia. 3
5
13
1
38


c.

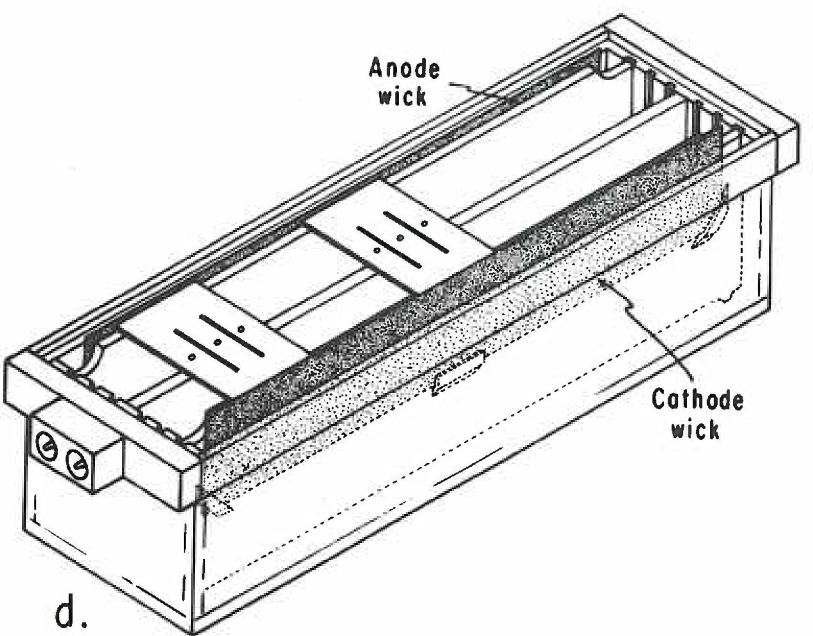
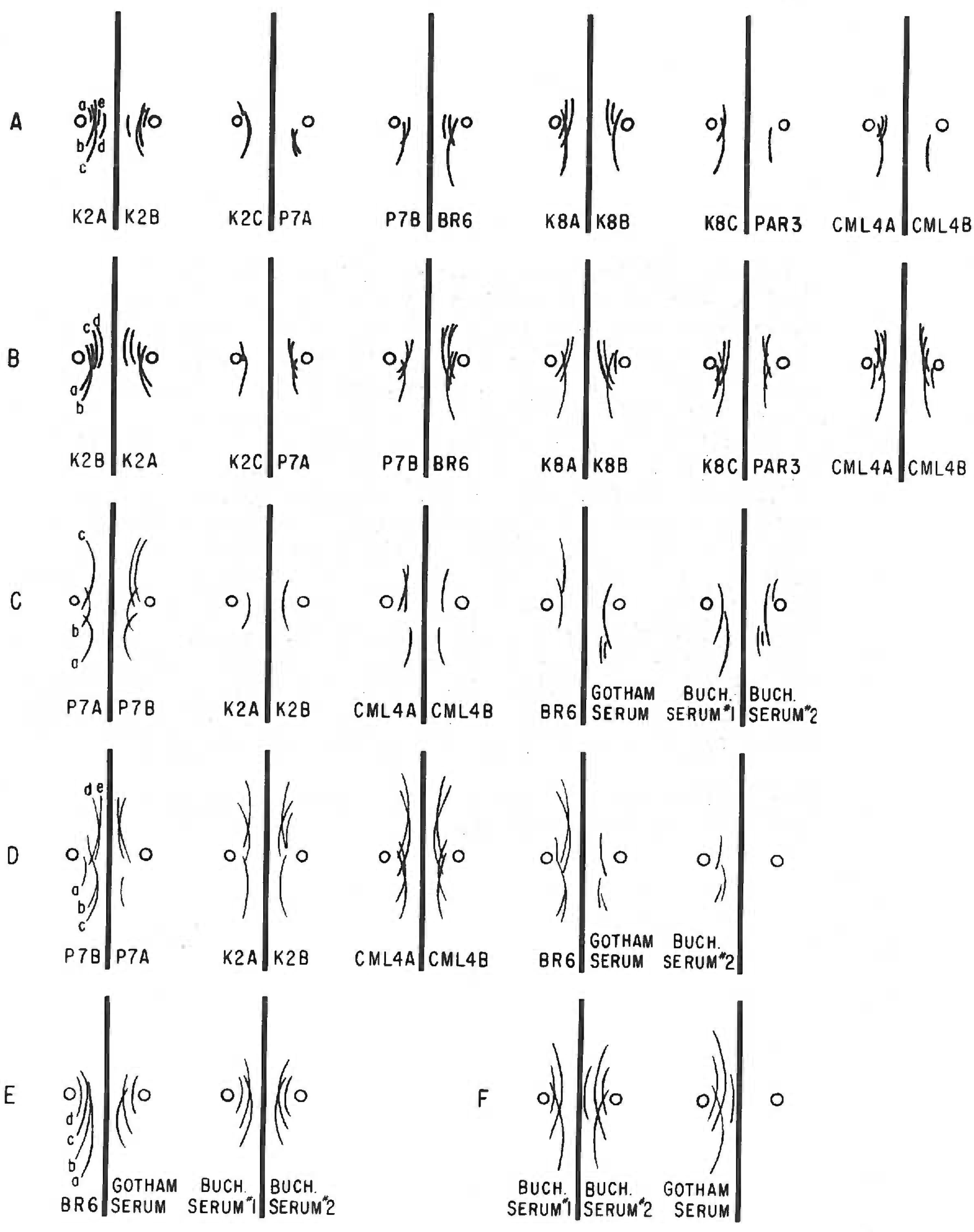
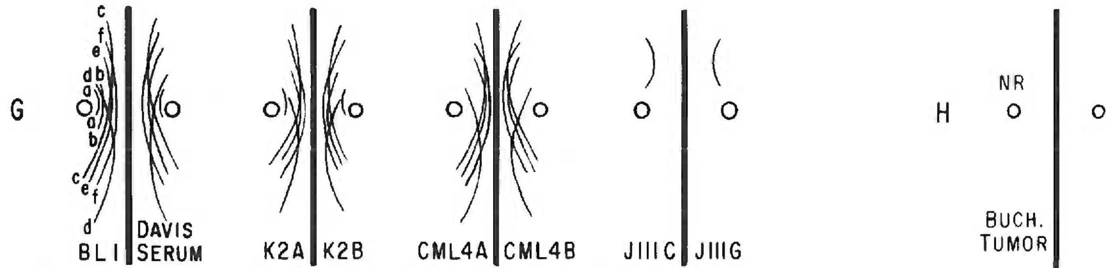

Anode wick
Cathode wick
d.

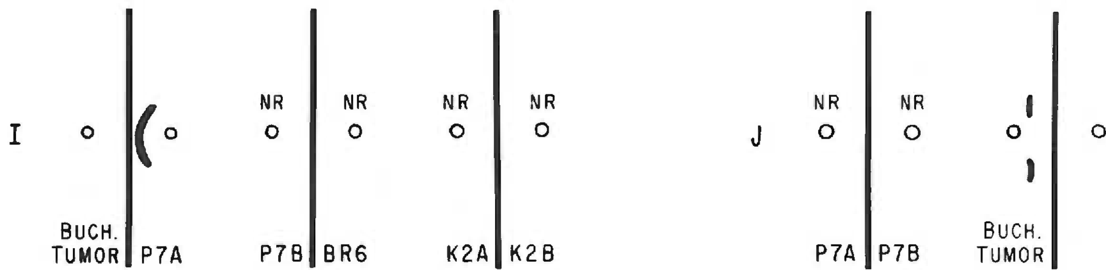
Figure 3

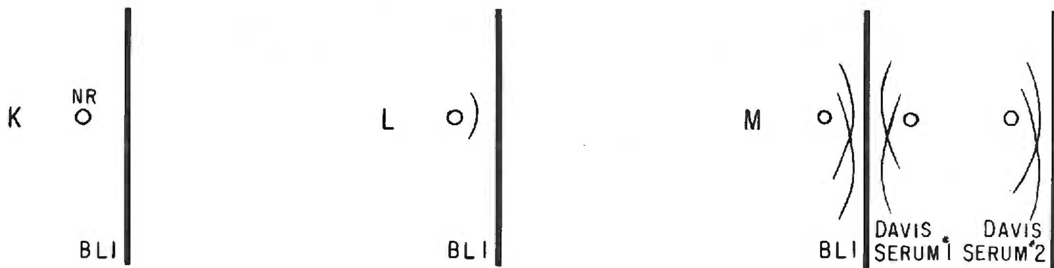
Summary of IEA reactions. Straight vertical lines represent antiserum troughs. Circles indicate antigen reservoirs. Arcs between the two represent observed antigen-antibody precipitates. Arcs in homologous reactions have been labelled with lower case letters. Antigens contained in the troughs have been designated by their code letters (Table 1) below each reaction zone. The upper case letters to the left of each series designate the antiserum used in the reactions of that series. They are as follows: (A) AbK2a, (B) AbK2b, (C) AbP7a (D) AbP7b, (E) AbBr6, (F) AbCML4a, (G) AbBL1 (H) Buch. Serum #1, (I) Buch. Serum #2, (J) Gotham Serum, (K) Davis Serum #1, (L) Davis Serum #2, (M) Gamma Globulin of AbBL1, (N) AbBL1 absorbed with Davis Serum #1, (O) AbBL1 absorbed with Davis Serum #2, (P) AbKC, (Q) AbKC5.

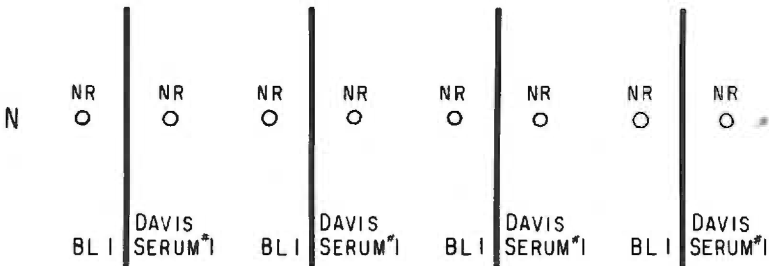
The anode is at the top of the figure and the cathode at the bottom.

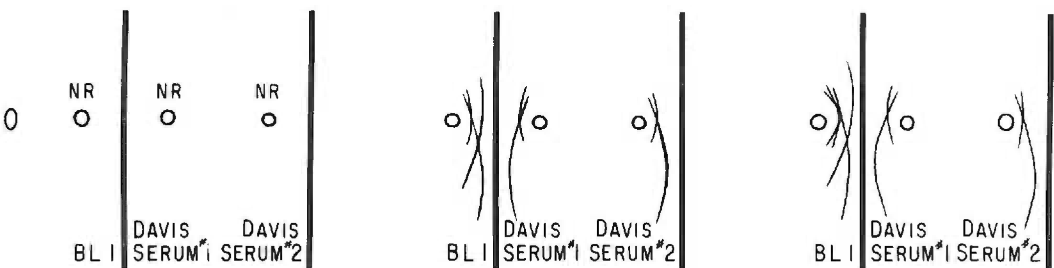

A
K2A K2B
K2C P7A
P7B BR6
K8A K8B
K8C PAR3
CML4A CML4B
B
K2B K2A
K2C P7A
P7B BR6
K8A K8B
K8C PAR3
CML4A CML4B
C
P7A P7B
K2A K2B
CML4A CML4B
BR6
GOTHAM SERUM
BUCH. SERUM #1
BUCH. SERUM #2
D
P7B P7A
K2A K2B
CML4A CML4B
BR6
GOTHAM SERUM
BUCH. SERUM #2
E
BR6
GOTHAM SERUM
BUCH. SERUM #1
BUCH. SERUM #2
F
BUCH. SERUM #1
BUCH. SERUM #2
GOTHAM SERUM


G
c
f
e
d
b
a
a
b
c
e
f
d
BL I
DAVIS
SERUM
K2A
K2B
CML4A
CML4B
JIIIC
JIIIG
H
NR
BUCH.
TUMOR


I
BUCH.
TUMOR
P7A
NR
NR
P7B
BR6
NR
NR
K2A
K2B
J
NR
NR
P7A
P7B
BUCH.
TUMOR


K
NR
BLI
L
BLI
M
BLI
DAVIS
SERUM*1
DAVIS
SERUM*2


N
NR
NR
BL I
DAVIS
SERUM*I
NR
NR
BL I
DAVIS
SERUM*I
NR
NR
BL I
DAVIS
SERUM*I
NR
NR
BL I
DAVIS
SERUM*I


O
NR
NR
NR
BL I
DAVIS
SERUM*I
DAVIS
SERUM*2
BL I
DAVIS
SERUM*I
DAVIS
SERUM*2
BL I
DAVIS
SERUM*I
DAVIS
SERUM*2

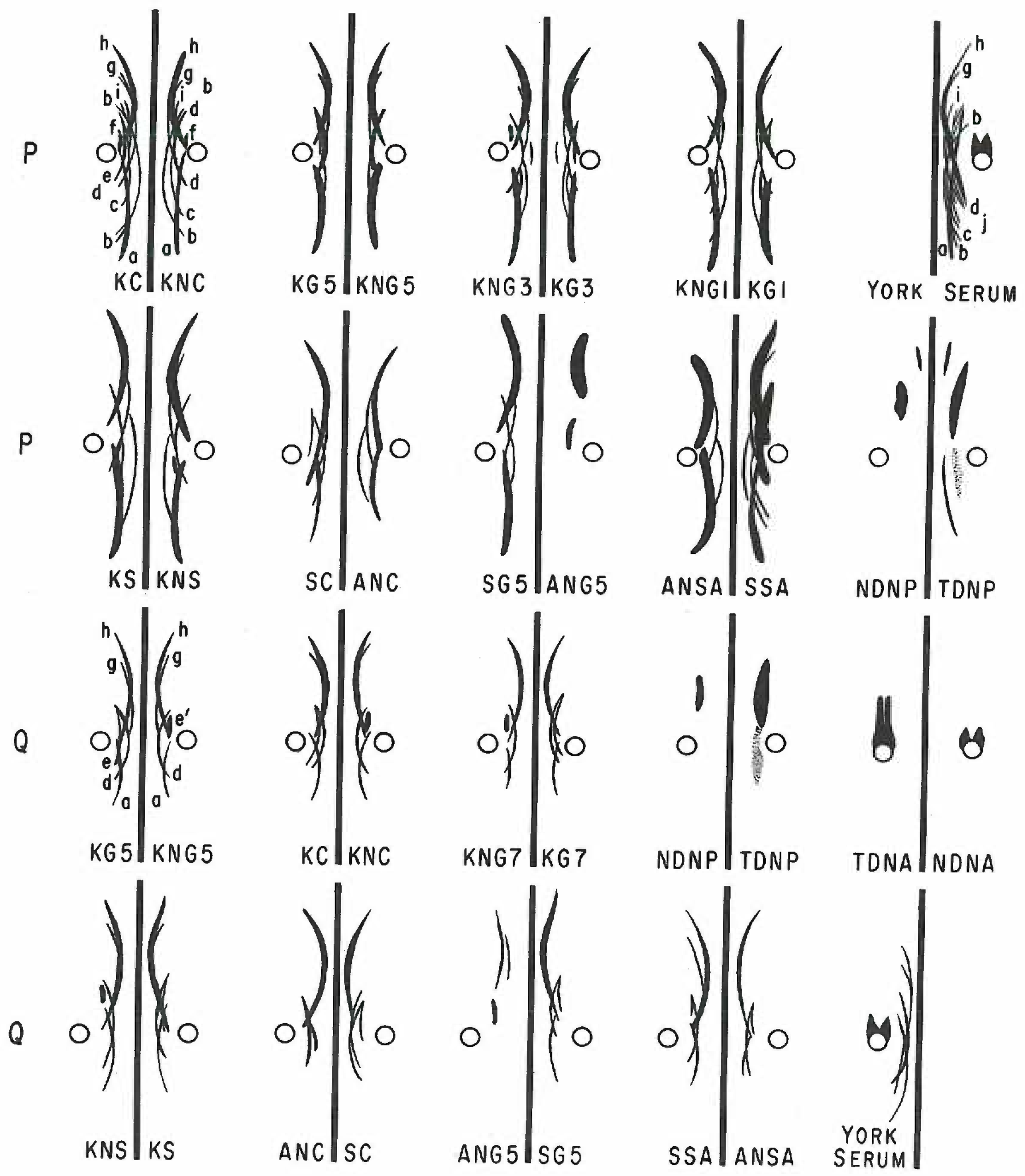
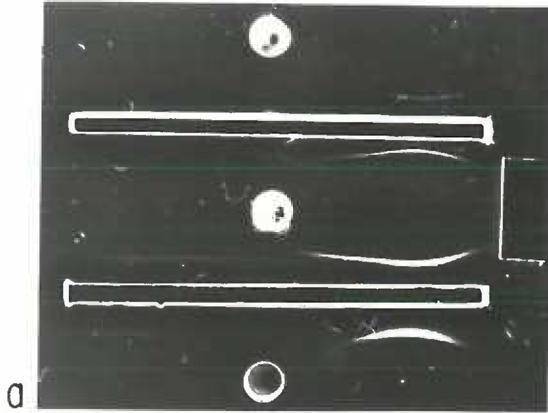
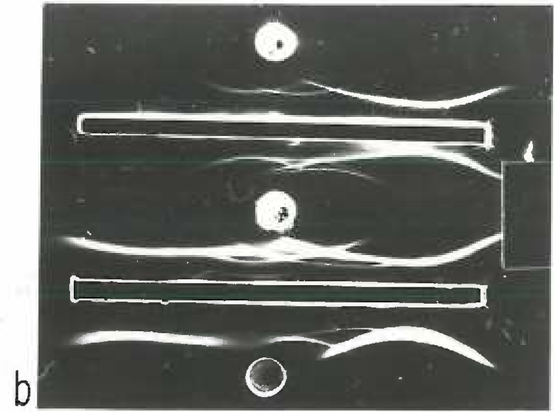

P
KC KNC
KG5 KNG5
KNG3 KG3
KNG1 KG1
YORK SERUM
P
KS KNS
SC ANC
SG5 ANG5
ANSA SSA
NDNP TDNP
Q
KG5 KNG5
KC KNC
KNG7 KG7
NDNP TDNP
TDNA NDNA
Q
KNS KS
ANC SC
ANG5 SG5
SSA ANSA
YORK SERUM

Figure 4

Changes that occur in an agar plate during its development. The times at which the photographs were taken (time zero is when the antiserum was added to the plate) are the following: (a) 5 hours, (b) 17 hours, (c) 41 hours, (d) 65 hours, (e) 74 hours, (f) 99 hours.


a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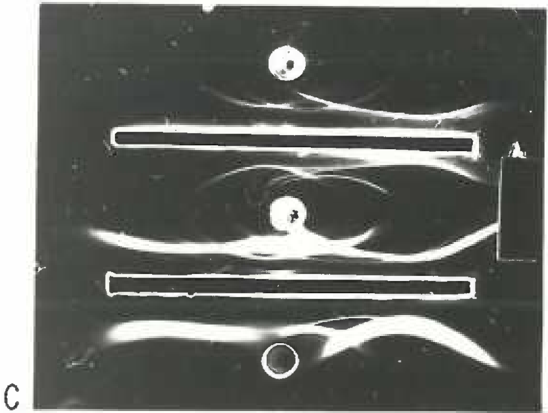
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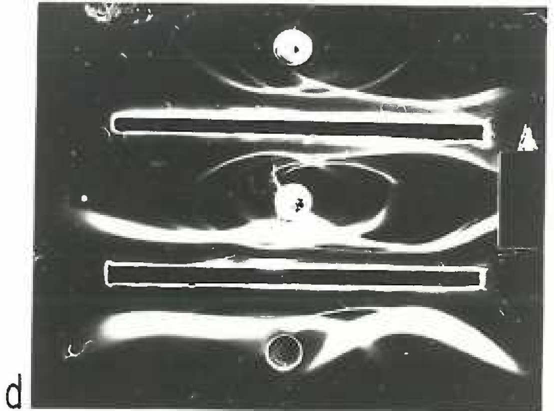
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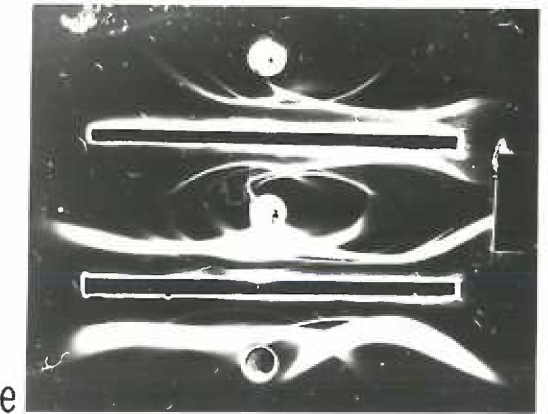
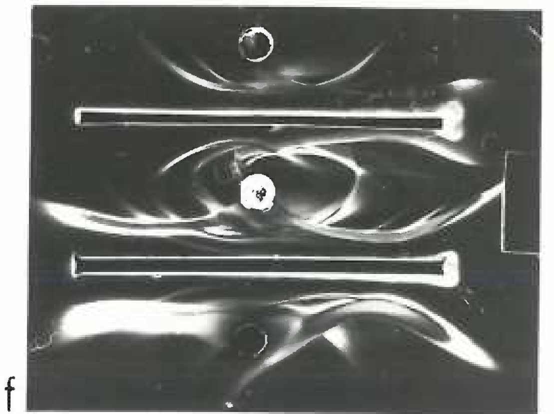

e


f