ALTERATION IN ULTRA-VIOLET ABSORBING CONSTITUENTS IN IN MURINE VIRUS-INDUCED LEUKEMIA

by

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INTRODUCTION

Statement of the problem

The implication of viruses as etiologic agents in a number of murine leukemias has contributed significantly to the present interest and work in the field of neoplasms. The development of several host-virus systems into controlled laboratory models has been of great aid to the field of cancer research. Examples of such host-virus models have been developed by Gross (1), Friend (2), Moloney (3, 4), and Stewart and Eddy (5). These models have yielded systems whose virus pools are stable, standardized, highly potent, and are suitable for systematic investigation over a period of time. In 1962, another host-virus system was described by Rauscher (6). This system, a newly isolated and highly infective murine leukemogenic virus, induced a dual type of disease. It was characterized by a rapid and extreme proliferation of principally erythrocytic and leukocytic elements, and in those surviving mice, the erythrocytopoiesis was followed by a lymphocytic leukemia beginning at 30-45 days post-inoculation.

The Rauscher model appears to be an ideal system to study the pathogenesis of leukemia because of the dual nature of the progression of the disease. With this system, it is possible to study the effects of the etiological agent upon the host system during the development of the two phases. A study was considered in which changes in the nucleic acid pool would be correlated with the pathogenesis of the disease.

Various investigators have shown that there is an alteration in the metabolism of nucleic acids subsequent to viral infection. Arnoff and Rafelson (7) have observed that there is a stimulation of the biosynthesis of nucleotides, i.e., the free nucleotide pool increases in infected

cells and tissues, upon viral infection. Craddock and Nakai (8) have observed that acute leukemic cells have a decreased rate of deoxyribonucleic acid synthesis. Rafelson (9) has shown, through chromatographic elution profiles, the effects of influenza virus upon the ribonucleic acid fractions of chick chorio-allantoic membranes. He observed no difference in the elution profiles of his controls but within virus-infected chorio-allantoic membranes he did get shifts and also an appearance of a new peak. In view of these observations it was thought of considerable interest to determine if the Rauscher leukemogenic virus would alter the pattern of the nucleic acid metabolism and to see if these changes could be correlated with the pathogenesis of the disease during the erythrocytic and lymphocytic stages.

Survey of the literature

Two basic properties common to all of the living organisms are their ability to 1) store, express, and transmit genetic information, and 2) to undergo mutation. These properties are dependent upon the chemical characteristics of a class of substances called nucleic acids, which are present in every living cell as well as viruses and bacteriophage. It is these substances, nucleic acids, which constitute the essential material of the genes and the apparatus by which they act. They contain in their structure the key or template for the normal growth and development of the organism. Thus, changes in their structure can lead to mutations, disease, and even death of the organism. Consequently, knowledge of the chemical and physical properties of nucleic acids is important not only from the standpoint of heredity but also for further understanding of the processes occurring at the cellular and organic level. This knowledge is important to understand the

processes involved in reproduction, cell division, tissue differentiation, and aging of organisms. It is because of this that so much stress has been placed on the field of nucleic acid chemistry and its relationship to various normal and abnormal life processes. The probable key may ultimately lie in the complete understanding and control of nucleic acid metabolism and its involvement in the life processes whereby man may be set free from disease. One of the fields which is presently being pursued by many investigators is the effect of host-virus relationship upon nucleic acid metabolism and the subsequent alteration in protein synthesis. A description of some of the work done in this area follows below.

Phage systems. Studies of the metabolism of phage-infected bacteria began in 1946. It was during that year that Cohen and Anderson (10) examined the rate of respiration of T2-infected E. coli cultures and hence, indirectly, of the overall energy supply furnished to the cell by its enzymatic system involved in uptake of oxygen, electron transport, oxidation, fermentation of carbohydrates, and the formation of energy-rich phosphate bonds. This study showed that T2 infection of bacteria stops immediately the formation of further respiratory enzymes, so that the respiration of (and thus energy flow into) the cells proceed at a constant rate for the entire latent period. Interference of this energy flow by respiratory enzyme inhibitors, such as cyanide, instantly stops the progress of phage development. At about this time Monod and Wollman (11) showed that phage-infected E. coli bacteria could no longer be induced to form their enzyme, β-galactosidase. Subsequent studies on the formation of other enzymes, of whose synthesis the uninfected cell is capable, showed a similar pattern, that is, infection by T-even phages caused the synthesis of bacterial

enzymes to come to a sudden and complete halt, and throughout the latent period, the activity of bacterial enzymes remained at a preinfection level (12, 13, 14). An apparent exception to the rule that synthesis of bacterial enzyme ceases upon T-even phage infection was discovered by Pardee (15, 16) who found there was a several fold increase in the activity of bacterial deoxyribonuclease following the infection of E. coli culture with T-even phage. It appears that the post-infection stimulation of deoxyribonuclease activity was actually the first observed instance of what is now called the "virus induced acquisition of metabolic functions" (17).

Cohen and Anderson (10), also in their first metabolic studies of T2-infected cells, measured the total amount of protein in the bacterial culture and found that overall protein synthesis in the bacterial cell is not inhibited by phage infection and continues at its pre-infection rate throughout the latent period. The phage infection has no immediate effect on the quantity of protein formed in the host cell, but only generates important changes in its quality.

It was through the works of Flaks and Cohen (17) and Kornberg (18, 19, 20) and others that the nature of the "early" protein essential for phage deoxyribonucleic acid replication was greatly clarified. They discovered a number of new enzymes concerned with the intermediary metabolism of the constituents of the T-even phage deoxyribonucleic acid. These enzymes are absent in the normal uninfected E. coli but make their intracellular appearance within a very short time after infection of other host bacterium by the T-even virus, thereby, representing a new species of protein molecules synthesized under the direction of the phage deoxyribonucleic acid. The following are some of the "early" proteins found during the initial phase of phage growth.

1. Deoxycytidylate hydroxymethylase (17, 21) catalyzes the addition of formaldehyde to deoxycytidine-5'-phosphate, which converts this deoxyribonucleic acid precursor nucleotide normally synthesized in the uninfected bacterium into the phage deoxyribonucleic acid component deoxy-5-hydroxymethyl-cytidine-5'-phosphate (or deoxy-HMC-5'-phosphate).
2. Kinase (19) which catalyzes the reaction between deoxy-HMC-5'-phosphate and adenosine triphosphate to yield deoxy-HMC-5'-triphosphate which acts as a substrate for the deoxyribonucleic acid polymerase.
3. Deoxyribonucleic acid polymerase (18) catalyzes the ordered copolymerization of the four nucleotide triphosphates into deoxyribonucleic acid polynucleotide.
4. Glucosyl transferase (19, 22) which catalyzes the action between uridine-diphosphate-glucose and the 5-hydroxymethyl group of HMC residues of intact deoxyribonucleic acid, to yield the glucosylated deoxyribonucleic acid molecules typical of the T-even phages.
5. Deoxycytidine pyrophosphatase (23, 19) catalyzes the conversion of deoxycytidine-5'-triphosphate to deoxycytidine-5'-phosphate. This enzyme is an antagonist for the synthesis of cytosine-containing deoxyribonucleic acid, by reversing the activating function of the normal kinase of uninfected bacteria which convert deoxycytidine-5'-phosphate to the triphosphate substrate of the bacterial deoxyribonucleic acid polymerase. Thus, the appearance of deoxycytidine triphosphates insures that no cytosine

finds its way into the deoxyribonucleic acid molecules synthesized after the onset of phage growth, unless the hydroxymethyl group has first been added to the 5-position of the pyrimidine residue.

6. Deoxycytidylic deaminase (20) is an enzyme which catalyzes the removal of the 6-amino group of deoxycytidylic acid, i.e., the conversion of the deoxycytidylic to deoxyuridylic acid.

7. Thymidylate synthetase (24, 25) catalyzes the addition of formaldehyde to the 5-position of deoxyuridine-5'-phosphate. Thus, the appearance of deoxycytidylic deaminase and thymidylate synthetase presents a new pathway for the metabolic biosynthesis of thymine in the infected cell.

In addition to the above enzymes, there were several enzymes already present prior to infection of the host which underwent significant increase in the course of intracellular phage growth. These enzymes include the deoxyribonuclease (15, 16) and a group of kinases (19, 26, 27) which catalyze the reaction of deoxythymidylic-5'-phosphate and deoxyguanylic-5'-phosphate to adenosine triphosphate to yield deoxythymidylic-5'-triphosphate and deoxyguanylic-5'-triphosphate respectively. These are substrates for the polymerase involved in the copolymerization of the deoxyribonucleic acid polynucleotide. It is believed from the observations made pertaining to the enzyme activity during post-infection that the increase in enzyme activity resides in new proteins whose functions resemble closely those of the autochthonous bacterial enzymes, but which upon closer examination, showed distinct behavioral differences from their bacterial analogues (26, 27, 28).

Cohen observed in his first metabolic experiments that ribonucleic

acid synthesis came to a sudden halt immediately following infection of the bacteria with T2 phage (29). This observation was in direct contrast to the observation that net protein synthesis continued at its pre-infection rate. Little, if any, increase of the amount of total ribonucleic acid per cell occurred during the remainder of the latent period, while intracellular multiplication of the virus was actively underway. This halt in ribonucleic acid synthesis reflected the termination of the formation of bacterial ribosomes which play an essential role in protein synthesis.

Ribonucleic acid is the principal phosphorylated constituent of E. coli bacteria and consequently the destination of most of the phosphorus assimilated by the cells during their normal growth. The blockage of ribonucleic acid anabolism upon phage infection thus allows synthesis of deoxyribonculeic acid to proceed at a much greater rate after infection rather than before without much necessity for the host cell to increase its phosphorus intake from the growth medium. Hershey (30) conducted an experiment using ^{32}P which was added to bacterial cultures only after their infection with T2 phage and found that during phage growth a small but significant amount of label entered the ribonucleic acid fraction of the infected cell. There is, therefore, some actual ribonucleic acid synthesis occurring within the infected cell. Volkin and Astrachan (31) showed further through the measurement of ^{32}P incorporation of each of the four ribonucleotides -- adenylic, guanylic, cytidylic, and uridylic acids-- that the label did not enter each of the ribonucleotides at the same rate at which the ^{32}P labeling of the ribonucleotides of the uninfected E. coli would occur under analogous conditions. This observation would indicate that the post-infection synthesis of ribonucleic acid concerned a species having a different

base composition from the overall ribonucleic acid of the host cell.

Tissue culture systems. Another model system which was studied in an attempt to elucidate the host-virus relationship involved the use of cell cultures. Studies dealt with, for example, were the metabolic events which transpired upon successful introduction of the virus nucleic acid into the host cell system.

Early studies of poliovirus effects on host cell ribonucleic acid failed to detect major changes during the infection. Salzman (32) reported that ribonucleic acid, deoxyribonucleic acid, and protein synthesis were inhibited in HeLa cells within 6 hours after poliovirus infection. Ackermann and co-workers (33) observed that the base-ratios of the ribonucleic acid-infected HeLa cells were the same as the control uninfected cells. But in 1961, Holland (34) showed, upon the examination of newly synthesized ribonucleic acid base-ratios in poliovirus-infected HeLa cells, that there was a marked shift in base-ratios. The base composition had shifted from a high guanine-cytosine content, which was characteristic of HeLa cell ribosomal ribonucleic acid, to high adenine values similar to the base-ratios of ribonucleic acid purified from poliovirus (35). The fact that there was a base-ratio shift in the newly synthesized ribonucleic acid without a significant change in the rate of ribonucleic acid synthesis suggested that the poliovirus was inhibiting the host cell ribonucleic acid synthesis as well as directing the synthesis of the viral ribonucleic acid type. It was shown later in subsequent work by Holland (36) that the host cell ribonucleic acid synthesis was drastically inhibited within several hours after infection by poliovirus. This demonstration was accomplished by infecting HeLa cells for several hours with poliovirus, then specifically inhibiting, by the addition of 10^{-3} M. guanidine, the virus directed

ribonucleic acid synthesis. Guanidine, at low concentration, was shown to prevent poliovirus multiplication and cytoplasmic changes and poliovirus ribonucleic acid synthesis without affecting the ability of HeLa cells to grow and its ability to synthesize ribonucleic acid (36, 37, 38). Holland (36) further observed that when guanidine was added immediately after infection with poliovirus, no virus-directed ribonucleic acid synthesis occurred, i.e., no infectious ribonucleic acid was replicated and no noticeable shifts in base-ratios were observed, while the host cell ribonucleic acid synthesis occurred at nearly the normal rate. But, if the infection was allowed to proceed for several hours before the addition of guanidine, then nearly all ribonucleic acid synthesis ceased. Holland thus felt that the virus infection inhibited host ribonucleic acid synthesis, and that the guanidine prevented the poliovirus-directed ribonucleic acid synthesis from replacing host ribonucleic synthesis which would normally occur.

It has been reported by Simon (39) and by Reich and Franklin (40) that neither deoxyribonucleic acid synthesis nor the integrity of preexisting deoxyribonucleic acid is necessary for the replication of the small ribonucleic acid viruses. The base-ratios of the deoxyribonucleic acid synthesized during poliovirus infection were identical to the normal HeLa cell deoxyribonucleic acid (41). But, there appeared to be a progressive inhibition of deoxyribonucleic acid synthesis in poliovirus-infected cells, and in cells infected by other small ribonucleic acid virus, i.e., myxovirus and Mengo virus (41, 32, 42, 43, 44).

Salzman (32) reported that the net synthesis of protein by HeLa cells was inhibited within 6 hours after poliovirus infection. It was shown, subsequently, by Zimmerman (42) and Holland (45) that the rate of protein synthesis dropped off rapidly following poliovirus infection.

Franklin and Baltimore (44) and Scholtissek et al. observed a similar depression of protein synthesis in mouse cells infected with Mengo virus and ME virus. Since poliovirus infection also inhibits ribonucleic acid synthesis, Holland (46) speculated that the inhibition of protein synthesis might merely be a depletion of messenger ribonucleic acid without replenishment by synthesis. This was tested indirectly by determining the effect of actinomycin D on HeLa cell protein synthesis (45). They observed that the actinomycin D rapidly inhibited nearly all of the HeLa cell ribonucleic acid synthesis, but also that the protein synthesis was depressed very slowly. Hence, it appeared that the messenger ribonucleic acid in these mammalian cells had a much longer half-life than the messenger ribonucleic acid in bacteria. In direct contrast, however, poliovirus infection caused a much more rapid decline in HeLa cell protein synthesis than did actinomycin, even though there was an apparent lag of several hours before ribonucleic acid synthesis was affected by the virus infection. Thus, it appears that the poliovirus infection inhibits the host cell protein synthesis by a more direct mechanism than by the inhibition of messenger ribonucleic acid synthesis.

A very important enzyme was recently identified in mouse cells and human cells which were infected with the Mengo virus and poliovirus, respectively, by Baltimore and Franklin (47, 48). This was a ribonucleic acid polymerase which appeared in the cytoplasm of cells infected with these small ribonucleic acid viruses. This enzyme is part of a particulate complex which seemed to carry its own template nucleic acid. It was postulated that this enzyme is the polymerase that is responsible for replicating poliovirus ribonucleic acid, since it is not found in normal cells, and its synthesis is inhibited by guanidine and other

specific poliovirus synthesis inhibitors (49). Further characterization of this enzyme and its template should provide invaluable information for the understanding of the biological mechanism involved in poliovirus replication.

Other host systems using different viruses were tested and their subsequent effects on metabolism were noted. Kun and his co-workers (50) investigated the influence of virus multiplication on the metabolism of the host tissue. The chorio-allantoic membranes of 12 days old embryonated White Leghorn eggs were infected with a suspension of a variant strain of avian canary pox virus. There was an observed increase in the utilization of glucose during the multiplication of the avian virus and also a marked increase in the levels of the enzyme, lactic dehydrogenase, in response to the viral infection. Arnoff and Rafelson (7) presented experiments which showed that the propagation of influenza virus was associated with an increased loss of radioactivity from the total ribonucleic acid fraction of chick chorio-allantoic membranes prelabeled with purine precursors but had no effect on the rate of incorporation of the purine precursors into that fraction. Since the rates of incorporation of purine precursors into the total ribonucleic acid of control and virus-infected membranes were the same, the increased loss of radioactivity from the ribonucleic acid of the infected tissue could not be explained on the basis of an increased rate of formation of the total ribonucleic acid in the infected tissue. The results were explained (7) or interpreted in terms of a "metabolic heterogeneity" of the ribonucleic acid. Rafelson (9) in a subsequent preliminary report presented information on the investigation pertaining to the metabolic behavior and constitution of various ribonucleic acid fractions isolated from control and virus-infected membranes. In

his investigation, he inoculated the chorio-allantoic membranes of 13 day old embryonated eggs with influenza A virus, PR8 strain, and grew these membranes in a defined media containing labeled compounds, glycine-1-^{14}C and adenine-8-^{14}C. The ribonucleic acid was isolated and purified, then chromatographed on a prepared cellulose ion-exchange column, ECTEOLA. The separated fractions of ribonucleic acid were then re-chromatographed using paper chromatography to determine the base composition. It was observed that in the case of the virus-infected tissue that there was a reduction of incorporated label of glycine into fraction IV and labeled adenine into fraction III which was contrary to the linear increase exhibited in all other fractions. Even though more work is necessary to show the significance of the differential rate of incorporation of the isotope into these ribonucleic acid fractions, it appears that the propagation of virus does result in an alteration both in the metabolic behavior and constitution of the ribonucleic acid which can be isolated from the chorio-allantoic membranes.

Vandevoorde, Hansen, and Nadler (51) cultured Osgood's tissue culture J-128 (a cell line originally derived from a patient with chronic granulocytic leukemia) on a simple medium not containing preformed purine or aminoimidazole carboxamide, to study the effect of azaserine on the metabolism of leukemic cell cultures. When hypoxanthine and aminoimidazole carboxamide were added to the culture media, these cells utilized these compounds as a source of nucleic acids, adenine and guanine. These compounds did not reverse the inhibitory effect of azaserine. From this observation the assumption was made that azaserine causes cell death by some mechanism(s) other than inhibition of de novo purine synthesis.

Other systems. Other "models" have been investigated in attempts

to understand the mechanism of nucleic acid and protein synthesis and the effects of viral infection and/or neoplasms upon these mechanisms. The model systems investigated have included mice infected with virus, humans with neoplastic conditions, rabbits, and other experimental laboratory animals. Many different techniques have been employed, such as, base content (9, 52, 53), base composition (34, 35, 41, 54), chromatographic elution profiles (7, 9, 55), molecular weight and other physico-chemical characteristics (56, 57) in attempts to detect peculiarities in the deoxyribonucleic acid and ribonucleic acids of leukemic cells and virus infected cells.

Some dynamic information has been obtained by studying the incorporation of isotopically labeled nucleic acid precursors (58, 59, 60, 61). It was observed that a high degree of incorporation was detected in all leukemic cells except the lymphocytes of chronic lymphatic leukemia. However, much of the intrinsic value of the results were lost due to the extreme cellular pleomorphism which results in different percentages of cellular types at various stages of maturation in both the normal and leukemic bone marrows. Gavosto, Maraini, and Pileri (62) believed that more reliable data could be obtained by comparing cellular types in normal and leukemic bone marrows. Autoradiography using tritium labeled compounds was employed in their experiment. With this technique, they investigated deoxyribonucleic acid metabolism using ^{3}H-cytidine and ^{3}H-DL-leucine as precursors. Bone marrows were obtained (6 normal donors and 8 patients with untreated acute leukemia) by sternal puncture. The tissue was incubated with the various labeled compounds, smeared onto plates, and grain counts then carried out. In this way they obtained data for a comparative study of deoxyribonucleic acid, ribonucleic acid, and protein metabolism in acute leukemic and

normal blast cells. They found a strikingly lower percentage of cells labeled in acute leukemic cells. This was interpreted as evidence of a decreased proliferative capacity, i.e., a decreased mitotic capacity of acute leukemia cells. This interpretation was supported by the observation that there also was an absence of significant difference in the mean grain counts of labeled cells in normal and leukemic myeloblast, indicating that the rate of deoxyribonucleic acid synthesis was essentially the same. Their data also showed a significantly lower uptake of labeled uridine, leucine, and phenylalanine in acute leukemic cells which was interpreted as a decreased ribonucleic acid and protein turnover. Gavosto et al. (62) felt that the existence of an interrelationship between ribonucleic acid and protein metabolism could be considered as evidence of a well-defined metabolic finality of the cell related to its specific functions (growth, synthesis of specific proteins, maturation, differentiation, etc.). The dissociation of ribonucleic acid and protein metabolism in the cells of acute leukemia could possibly be related to the well-known incapacity of these elements to differentiate and to mature; this, together with their lower proliferative capacity, seems to constitute the most important functional defect of these elements.

Siegel and Kuusi (63) analyzed the purine and pyrimidine composition of the cytoplasmic pentose nucleic acid (ribonucleic acid) of normal and poliomyelitis virus infected mouse brains. In their investigation, Webster strain white mice were inoculated intracerebrally with a suspension of MEF_1 strain (Type 2) poliomyelitis virus. The infected brains were harvested periodically from animals showing paralytic symptoms of central nervous system involvement. A comparison of the composition of normal and infected mouse brain ribonucleic acid showed a difference in

the uracil content, which appeared higher in the infected than in the normal mice. They postulated from their results that this finding may indicate an enhanced propensity of brain cells in the infected animal to synthesize ribonucleic acid or a new type or that in the process of cellular degeneration, a molecular species or residue of ribonucleic acid richer in uracil was broken down less rapidly than other molecular species of the cell.

Other phases of metabolism were studied following infection of mice with poliomyelitis virus, strain MEF_1 (Type 2) by Siegel and Hughes (64) and Siegel, Hughes, and Tolbert (65). In these experiments, the effect of virus infection upon glucose oxidation and acetate metabolism were studied. In the case of glucose oxidation there was a slight decrease in the infected animals, whereas, in contrast, there appeared a slight increase in acetate metabolism. It was concluded that the interesting phenomenon was not so much the magnitude of change in the infected animals, but the lack of any large effect. They observed that as long as the animal was alive, that their intermediary metabolic pathways proceeded at approximately the normal rate as was measured by these gross respiratory studies.

Wenner and co-workers (66) studied the effects of oncogenic and non-oncogenic viruses, such as, Newcastle, vaccinia, and Columbia-SK, and observed that there was an increase in lactic dehydrogenase activity. Weaver, Weimar, Roberts, and Siegel (67) studied the pattern of lactic dehydrogenase production in BALB/c mice infected with a murine leukemogenic (Rauscher) virus. They reported that the dehydrogenase activity increased progressively with time in the infected animals to a value as high as 22 times that which was observed in the control animals. Also it was observed that the increased lactic dehydrogenase activity was

coterminous with increased spleen weights and increased nucleated cell counts. Thus, the increased activity of the enzyme closely paralleled the pathogenesis of development of the disease caused by the leukemogenic virus.

Another interesting piece of work was reported by Baldessarini and Carbone (68). They had shown that the white blood cell preparation from patients with chronic myelocytic leukemia had more S-adenosyl-methionine than in normal peripheral white cells or thoracic-duct lymphocytes. He also corroborated previous reports that the cyanocobalamine levels (vitamin B_{12}) were elevated in the serum of these patients suffering from this disease. It has also been observed that the folic acid level increased in leukemic cells (69). These compounds, cyanocobalamine, folic acid, and S-adenosyl-methionine are all involved in transferring methyl groups. Methyl group transfer is important in the synthesis of the methylated pyrimidine, thymine. Baldessarini felt that the elevation of these compounds should warrant extensive evaluation, biochemically and metabolically of the relationship of biological transmethylation with respect to leukemia.

Krupnick and Rosenkrantz (70) investigated the nucleic acid content in spleen and neoplastic tissue of several rat strains. Two mammary adenocarcinomas designated R-35 and MT/W9A, and a mammary fibroadenoma, referred to as the C-tumor, in addition to a lymphocytic leukemia, WR-6, were studied. The R-35 mammary adenocarcinoma and C-tumor mammary fibroadenoma were sublines of a spontaneous mammary fibroadenoma originally found by Huggins (71) in a Sprague-Dawley rat. The R-35 and C-tumor were transplanted into female rats, 41-51 days old, in a similar fashion. One or two slices (10 X 10 X 1 mm) of tumor were implanted subcutaneously in a suprascapular or suprapelvic position or both. The lymphocytic

leukemia, WR-6, arose spontaneously in Furth's WFu/SCF (Wistar-Furth from A. R. Schmidt) rat strain (77). They implanted a mince of donor tumor tissue subcutaneously and laterally into 45 days old female Wistar-Furth rats. A tumor mass from this implantation was evident in 5-7 days post-inoculation and white blood counts, primarily lymphocytes, were markedly elevated. The mammary adenocarcinoma, MT/W9A, was originated in Furth's inbred Wistar rats. The MT/W9A was implanted similarly to WR-6. Their results showed that the nucleic acid ratio (RNA/DNA) for all mammary neoplasms increased. In the case of the C-tumor, the increment was due to an increased ribonucleic acid content. On the other hand, both ribonucleic acid and deoxyribonucleic acid levels in the R-35 and MT/W9A tumor tissue were found to be higher than in normal mammary tissue. The deoxyribonucleic acid and ribonucleic acid levels in the spleens of leukemic rats (due to WR-6) were well within the range of values obtained from control animals. The R-35 implanted rat spleens disclosed normal values of nucleic acid as compared with control rats. The animals implanted with MT/W9A tumor did not show alterations of deoxyribonucleic acid levels in the spleen. They concluded that despite the lack of an observable interplay between the tumor and host spleen, nucleic acid measurements suggested that the RNA/DNA ratios may be helpful in characterizing the tumor system involved since the RNA/DNA ratio progressively increased with survival time. For example, the rapid onset of the WR-6 was characterized by a RNA/DNA ratio of 0.73 while the C-tumor system reached a ratio of 2.82. The other mammary neoplasms with intermediate survival times yielded ratios between 0.90 and 1.27.

A quantitative cytochemical investigation of deoxyribonucleic acid, ribonucleic acid and protein in lymphoid cells from normal, preleukemic, and leukemic mice was conducted by Gahrton, Halbicht, and Warren (73).

The animals used in this study were: 1) C57BR/cd, 2 months old. This group was referred to as normal. The leukemia incidence in this strain was < 1 percent (0/164) in breeding mice; 2) AKR/JAX, 2 months old; 3) AKR/JAX, 6 months old; 4) AKR/JAX, primary lymphoid leukemia. Apart from these groups, one C57BR/cd mouse with a primary Gross Passage A virus-induced lymphoma (74) was studied. Thymus cells were investigated in all cases as well as lymph node cells from the first group and from the Gross virus-induced leukemia. The deoxyribonucleic acid and total nucleotide content of the cells were measured in a rapid scanning microspectrophotometer. The AKR leukemic cells were found to differ from the normal in the following respect. The majority of leukemic cells had a pronounced increase of ribonucleic acid and protein without a comparable increase of deoxyribonucleic acid. Also, the variation in all three parameters were greater in the leukemic cell populations than in normal thymus cells. They explained the augmented variability of deoxyribonucleic acid, ribonucleic acid, and protein contents in the leukemic cell populations either as a dedifferentiation, as an augmented momentary variation, as the presence of non-participating cells, or as a combination of all three factors. There are strong indications that the etiology of the AKR leukemia is viral as demonstrated by the repeated isolation of leukemogenic virus from AKR tissues of embryonic or leukemic origin (1, 75). Also, there is an immunologic cross-reaction between the AKR leukemias and leukemias induced by the Gross Passage A virus (76).

A pronounced increase in thymidine kinase activity has been observed following infection of primary cultures of murine cells with polyoma virus (77, 78, 79, 80). Kit and his associates (81) investigated the nucleic acid metabolism in Swiss mouse embryo and Swiss mouse kidney

cells infected with polyoma virus, strains SP208 and TR-6. The investigators observed a marked enhancement of phosphorylation of thymidine-^{3}H and deoxyuridine-^{3}H in primary cultures of murine cells 16-48 hours after polyoma virus infection. The thymidine kinase activity, as measured by the phosphorylation of the tritiated compounds, of the infected cells attained values of up to 55 times that of the non-infected cells. They observed that the increased thymidine kinase activity facilitated deoxyribonucleic acid biosynthesis in the polyoma virus-infected cultures and postulated that this may also occur in vivo in virus-infected animals. Three functions for thymidine kinase were suggested:

1. Thymidine kinase may counteract the effects of dUMP and TMP phosphatase activities since nucleotide-5'-phosphatase activity is extremely active in some tissues, thereby depleting the pools of dUMP needed for TMP synthesis and the pools of TMP needed for TTP and deoxyribonucleic acid synthesis.
2. By helping to maintain the pools of TMP, thymidine kinase may function in activating and stabalizing TMP kinase.
3. Thymidine kinase may function in "fixing" some of the thymidine circulating through the host blood stream.

Thymidylate kinase activity also was observed to increase in polyoma virus-infected cultures, but uridine kinase and thymidylate phosphatase activities were not seen to increase.

Silber and his group (82) studied the effect of a murine leukemia on enzymes of one-carbon metabolism and on phosphomonoesterases. In their studies, DBA/2 mice were infected with the Friend virus (2) and the effect upon the activity of two groups of enzymes were investigated.

In one group, the levels of three one-carbon metabolism enzymes, thymidylate synthetase, dihydrofolic reductase, and the formate activating enzyme showed in the spleen an increase ranging from 3-10 times their initial low activity during the first two weeks of the disease. The peak levels attained in the leukemic spleen were approximately the same as those found in subcutaneous tissue of the same type. The histological studies suggested that the increase in activity paralleled the proliferation of neoplastic cells. The other group was the measurement of alkaline phosphatase levels. The alkaline phosphatase level of the leukemic spleen was decreased to about one-half of its normal activity. The activity of this enzyme in the subcutaneous tumors derived from leukemic organs were relatively low. However, the alkaline phosphatase activity of leukemic livers increased up to 15-fold from the initial, whereas, the enzymes of one-carbon metabolism were not affected. They, on basis of histochemical studies and splenectomy, did not believe that the increase of hepatic alkaline phosphatase was directly related to the presence of tumor cells in the liver or to splenic enlargement.

Rauscher Virus

In 1959, Rauscher began studies on a virus induced leukemia of adult random-bred Swiss mice which had been reported previously by Schoolman, Spurrier, Schwartz, and Szanto (83). Rauscher (6) produced one lymphocytic tumor in 508 BALB/c mice eleven weeks after inoculation of a filtrate of the Schoolman-Schwartz Swiss mouse lymphoblastoma. This lymphoblastoma appeared to be the source of the virus which was subsequently isolated. An extract of the tumor tissue of the ninth serial whole cell transplant produced lymphocytic leukemia in three mice out of forty-six that were inoculated. Tissue from these three mice

were used to make a cell-free extract. This extract, when injected into normal BALB/c mice, produced virtually 100 per cent contraction of the disease. Siegel and his associates (84) described the disease to be primarily an erythroleukemic response in mice to this virus which was reminiscent of the Di Guglielmo's disease in man (85).

Description of the disease. The latent period, the time following inoculation to the time at which the spleen is palpable, in different age groups were found to be approximately equal. The death rate is dependent upon the age of the mice at the time of inoculation, that is, the death rate decreases as the animal age increases. Macroscopic findings showed that the spleens were easily palpable within 10-15 days post-inoculation, with death beginning in the mice between 28-37 days post-inocualtion. Generally, the inoculated mice had increases in spleen weights which were 30-40 times the spleen weights found in normal mice of comparable age. The enlarged spleens were spongy in texture and contained enormous sacs of blood. Hepatomegaly was noted with a 2-4 fold increase in weight at the time of death. Some slight enlargment of the peripheral lymph nodes were seen while the thymus showed no gross enlargment. However, survivors of the early mortality peak associated with hepatosplenomegaly did develop enlarged peripheral lymph nodes and thymus. Spleens of the surviving group, in direct contradistinction with the spleens of the non-survivors, were firm in texture and did not enlarge. A chronic course followed in the surviving mice with mortality occurring between the 62 to 90 days.

Histological findings. The microscopic findings of various organs revealed: 1) extreme numbers of nucleated erythrocytes and erythroblastic cells in the red pulp of the spleen; 2) replacement of much of the red

pulp by recent hemorrhage in older areas of hemorrhage where organization had taken place; 3) many nucleated red cells are seen in the sinusoids of the liver; 4) accumulations of granulocytes in the medullas of lymph nodes; 5) in those animals which did not develop lymphocytic leukemia, the thymus underwent atrophy with a decrease in thymocytes; 6) and in the lymphocytic leukemic mice the thymus, lymph nodes and spleens were infiltrated by a large mononuclear basophilic cells. Lymphocytic leukemia cells and an increase in the numbers of the nucleated red cells were seen in the peripheral blood smears of the leukemic mice. Rauscher and his colleagues testing the efficiency of several routes of inoculation of the virus found that the mice inoculated via the interperitoneal or intravenous routes had measurable infection in one-third to one-fourth the time taken by other routes. Electron microscopic examination of the diseased tissue presented no evidence that two viruses were present to account for the two separate phases of the disease. The virus is very stable and has a high leukemogenic potency when frozen at -70°C. for five months. Rauscher provided data which showed that there was no loss of potency of the virus after incubation with rabbit antiserum prepared against the leukemogenic viruses of Friend, Moloney, and Schoolman-Schwartz (6). Ziegel and Rauscher (86), in a later study, using a negative staining technique, showed by electron micrographs that 80 per cent of the particles had tail-like projections extending from the "head". The "head" was approximately 120 mμ in diameter and was angular with a hexagonal shape. In 1965, Mora, McFarland, and Luborsky (87) reported the first successful extraction of an intact ribonucleic acid from the Rauscher murine leukemia virus in amounts sufficient for characterization by physico-chemical means. In their study, electron micrographs of the virus showed that the virus population recovered

was virtually pure "C-type" virus particles (88) characteristic of the murine leukemia virus (89). Other observations, i.e., the diameter of the particles being around 100 mμ and that many of the particles having a "tail-like" elongated appendices, confirmed the report made by Ziegel and Rauscher (86). From their ribonucleic acid studies, they concluded that the ribonucleic acid occurring in the infectious Rauscher murine leukemia virion had a sedimentation coefficient of 73S in 0.2 M salt, was in the single-stranded form and as a single molecule had a molecular weight of about 13 X 10^6. To the best of their knowledge, it was believed that this virus contained the largest ribonucleic acid ever observed. Propylthiouracil and transfusion modifications of the effects of Rauscher virus in BALB/c mice experiments were conducted in 1965 by Dunn, Malmgren, Carney, and Green (90). Because the reaction in the mouse appeared to be primarily erythroblastic (84), procedures known to inhibit erythrocyte production were tested. Two which were definitely effective were propylthiouracil feeding and blood transfusions. They noted that when these procedures were used, the survival time was greatly prolonged, approximately two-fold; and the spleen, though enlarged, was firm and compact and not spongy as in untreated virus-infected mice. In the treated mice the erythroblastic proliferation characteristic of the Rauscher virus reaction was inhibited but never completely suppressed.

Immunological studies of the virus have been carried out by Fink and Rauscher (91) and by Siegel and Morton (92). In the latter, the authors noted a depression in the immune response to bovine serum albumin with leukemogenic virus infection.

Cytological studies and histochemical enzyme analysis have been carried out chronologically on individual BALB/c mice following inocu-

lation with Rauscher leukemogenic virus by Mathias, Hunt, Lawrence, and Siegel (93) and by Mathias, Hunt, Florey, and Siegel (94). In their studies, a marked increase in cytoplasmic blebbing of lymphocytes was noted during the development of the disease along with the appearance of V-cells (95). There was a great increase in LDH activity in the lymphocytes, V-cells, and immature cells of the erythrocytic series as the disease progressed. The majority of lymphocytes from infected animals showed an increased acid phosphatase reaction, while the alkaline phosphatase activity remained negative as in the normal controls.

In a review of the literature, no reports were found which discussed the effect of Rauscher murine leukemia upon nucleic acid metabolism and the subsequent effect upon protein synthesis. The Rauscher virus warrants much more study, which may eventually lead to a better understanding of some of the fundamental problems of blood formation and leukemogenesis.

MATERIALS AND METHODS

Animals

The animals used in this investigation were female Balb/c mice obtained from the Simonson Laboratory, Gilroy, California. The approximate age of the mice at the beginning of the experiment was five weeks. The animals were divided into groups of eight and were housed in stainless steel cages. They were given a standard diet of Purina laboratory chow. Individual animals were numbered using a color code system to correspond to the various dye colors.

Experimental groups

The information obtained in this investigation was acquired from three groups of mice. Groups A and B consisted of 8 animals each and Group C, the control, likewise consisted of 8 mice. Groups A and B were inoculated with the Rauscher leukemogenic virus. Each of the three groups, A, B, and C, were housed separately. Blood was drawn from the ophthalmic venous plexus at weekly intervals and blood differentials performed to determine the progress of the viral infection until the termination of the experiment. Forty-eight hour urine samples were collected each week during the course of the experiment in order to investigate any temporal changes in the nucleic acid pools.

Virus suspension

The suspension of spleen extracts of virus was originally obtained from Dr. Rauscher. This virus pool was maintained in this laboratory by serial passage in Balb/c mice. The virus suspension inoculum was prepared in the following manner. The infected mice were maintained until they showed evidence of gross splenomegaly by palpation. At this time the mice were sacrificed by cervical dislocation and the

spleen excised and weighed under sterile procedure. The pooled spleen tissue was homogenized in an all-glass homogenizer, using Hanks balanced salt solution with added antibiotics. The homogenate was centrifuged in a refrigerated International PR-2 centrifuge at 2,500 rpm at 4°C for twenty minutes and the supernatant then drawn off and centrifuged a second time to obtain a cell-debris free spleen extract suspension. Diluent was finally added to produce a 40 per cent suspension. The 40 per cent virus suspension was routinely stored in sealed glass ampules at -70°C in a Revco freezer until time of inoculation.

Inoculation of animals

The experimental mice were inoculated intraperitoneally in the lower right abdominal quadrant with 0.2 ml of a 15 per cent virus suspension. The virus suspension used in this study was an eighth Balb/c serial passage.

Collection of urine samples

(a) Urine collection apparatus. A common method used in collecting metabolic products, fecal or urine, is through the use of a metabolism cage. There are available many types of metabolism cages for use in experimental studies of metabolism. Most of the available metabolic cages suffer from a common problem which was not tolerable in this type of investigation. The problem is one of urine contamination by the feces, either through feces dropping into the collected urine or by urine flowing through the accumulated feces. Contamination of this nature could greatly alter the composition of the urine sample. To preclude this source of error a metabolism cage was designed and constructed in this laboratory (Appendix A-a) which would minimize contact of urine and feces and assure almost complete separation of

of feces and urine.

A regular 7-in x 7 1/2-in x 10-in stainless steel animal cage, as modified and shown in Appendix A-b, was placed upon the metabolism collection unit.

(b) Urine collection procedure. The mice from each group, A, B, and C, were placed into the cages 24 hours prior to actual collection of the urine. This was to allow the mice to become acclimated to their new environment since it was previously observed that mice would suffer from diarrhea or more moist feces when introduced to new surroundings. The acclimatization period was found to be essential for obtaining good separation of the urine from fecal matter. Twenty-four hours was observed to be adequate for this purpose.

Following the acclimatization period the collection apparatus was thoroughly cleaned with Virac and water, dried, and the urine sample holder put into place. The urine was then collected for 48 hours. This time period was chosen to assure sufficient urine collection for the chromatographic analysis employed in this investigation. At the end of the 48 hour collection, the mice were returned to their cages. The mice were allowed to feed and water at ad libitum during the acclimatization and collection period. The urine which had dried and accumulated on the collection mechanism was harvested by washing down with 0.5 ml of 0.85% NaCl solution (Merck Biological Reagent). This was was pooled with the urine collected in the urine sample holder. The collected urine sample was then drawn into a disposable tuberculin syringe (Monoject, Cat. no. 501-TB) and the volume of collected urine measured and recorded. The urine was subsequently transferred to a 3 dram screw cap vial, labeled, and stored at -5°C until the time of analysis.

Preparation of urine for assay

The stored, frozen urine was allowed to thaw at room temperature. Upon thawing, the urine was treated with 0.5 N $HClO_4$ (Merck) at room temperature for 15 minutes. The urine was neutralized with 5.0 N KOH (Merck), adjusted to pH 5.8 and then centrifuged in a modified Wifug, Rastgeldi type, centrifuge (Appendix B) at 3,000 rpm for 15 minutes to remove all precipitates. The supernatant was drawn off with a disposable tuberculin syringe (Monoject), the volume measured and transferred to a 3 dram screw cap vial. The optical density of the $HClO_4$-treated urine was determined by the following procedure: 100 microliters of the treated urine was drawn up with a Hamilton, Model 710, microliter syringe and transferred to a 50 ml Pyrex volumetric flask and diluted to volume with glass distilled water. Routinely, the same 50 ml volumetric flask was used in optical density determination of all samples. The absorbance of the diluted urine sample, 1/500 dilution, was determined at 260 mμ using a Beckman, Model DB, spectrophotometer. The amount of urine sample routinely used for analysis was 168 O.D.U. This value was chosen because of the absorbance limits of the spectrophotometer.

Collection of blood samples

All mice, control and infected, were followed serially during the course of the experiment. Hematological data was obtained through weekly blood drawings from the ophthalmic venous plexus. This technique could be repeated any number of times with infrequent tissue damage or infection. The animal was laid on its side with the head held firmly between the thumb and middle finger. A micro-heparinized tube (diameter 1.4-1.6 mm, length 75 mm, Chase Instrument Corporation) was then placed at the lower corner of the eye orbit and pushed into the ophthalmic

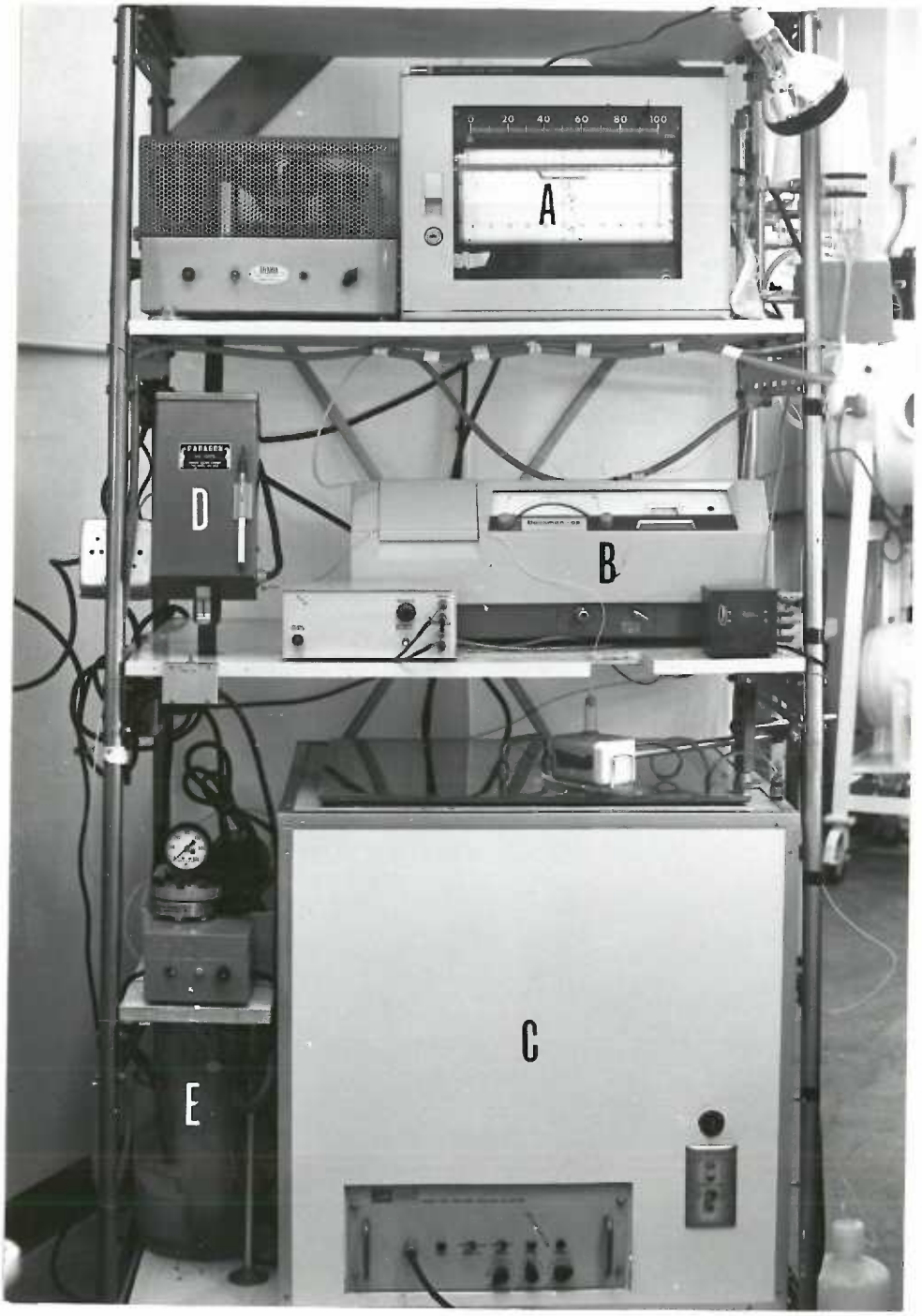
venous plexus which lines the back of the orbit. As a result of rupturing the venous capillaries, the reservior formed by the orbital cavity becomes filled with blood and the inserted tube becomes filled by capillary action. Slides for differential determination were made by smears of a drop of blood from the capillary tube on a glass slide.

Analyzing system

(a) Double-beam monitoring system. The analyzing system used in this investigation (Fig. 1) was essentially the nucleotide analyzer described by Anderson (96). A detailed description of the spectrophotometric column monitoring system is presented in Appendix C. Anderson's system was slightly modified for use in this laboratory. A refrigerated fraction collector was substituted for the regular open-air fraction collector and the wavelength selector mechanism was modified from Anderson's pneumatic air-piston type to one of an electronic design (Appendix D).

Essentially, this is a chromatographic method for the separation of nucleosides, nucleotides, purine and pyrimidine bases using Dowex 1-X8 resin columns 0.9 x 115 cm in length. Absorbance at 260 and 280 mμ at an optical path length of 1.0 cm are continuously recorded using a modified double-beam spectrophotometer. Identification of the various nucleic acid components are based upon column retention volume and absorbancy ratios derived from the recorded 260 and 280 mμ readings. These parameters are utilized in identification of peaks obtained from chromatographic elution profiles of experimental samples and comparing these same parameters with the chromatographic elution profiles obtained from known standards chromatographed under similar conditions.

(Figure 1). Photograph of the double-beam continuous column monitoring system used to obtain chromatographic elution profiles of urine samples. Major components seen are the recording potentiometer (A), modified Beckman DB spectrophotometer (B), refrigerated fraction collector (C), interval timer (D), and constant temperature circulating bath (E)


A
B
C
D
E

(b) Preparation of column. Dowex 1-X8 resin 200-400 mesh (obtained from Dr. Paul Van Dreal[1]) was treated with 1.0 N HCl and 1.0 N NaOH. The resin was then repeatedly washed with glass distilled water until all Cl^- was washed out. Following this washing, resin fines were removed by allowing the bulk of the resin suspension to settle, and fines remaining in the supernatant were removed with a Pasteur pipet attached to an aspirator. Repeated resuspension in glass distilled water, settling, and aspiration removed most of the fines. The resin suspension was then stirred in 3 M glacial acetic acid to ensure removal of CO_2 bubbles before packing the column. The resin was allowed to settle and the 3 M acetic acid was decanted off. The settled resin was suspended in 0.15 M acetic acid-sodium acetate buffer, pH 4.4, for packing. Approximately 10 ml of 0.15 M acetic acid-sodium acetate buffer, pH 4.4, was poured into the column. The resin slurry was then poured into the column and packed under pressure with a hydraulic positive pressure pump (Milton Roy minipump) at 100 per cent stroke length. When the majority of the resin had settled in the column, the supernatant was removed by means of a polyethylene tube connected to a 50 cc disposable plastic syringe. The column was again filled with the resin slurry and the packing procedure repeated until a column bed 110-120 cm in length was obtained. After packing, the column was washed with 1200 ml of 0.15 M acetic acid-sodium acetate buffer at pH 4.4.

(c) Production of gradients. Three 600 ml Pyrex cylinders were connected in series, with a magnetic stirrer in the cylinder connected

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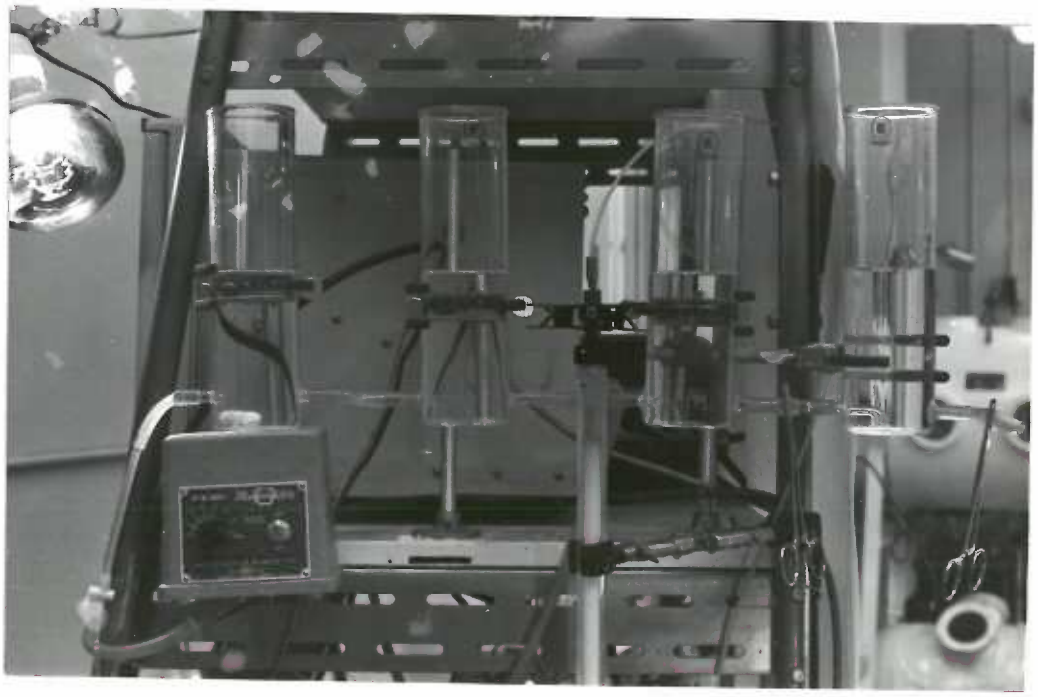
to the pump (Fig. 2). The first cylinder contained 600 ml 0.15 M acetic acid-sodium acetate buffer and the second and third 3.0 M acetic acid-sodium acetate buffer, all at pH 4.4, to form an 1800 ml convex gradient. A 250 watt infra-red (Ken Rad,special service infra-red) lamp over the first cylinder warmed the buffer to 45-50°C in order to reduce the level of dissolved gases and prevent air bubbles in the spectrophotometer flow-cell cuvette. A drop of N-octanol was also added to each cylinder to minimize trapping of air bubbles in the cells.

(d) Calibration of flow rates. The flow rate of the column was determined at various per cent stroke lengths of the pump by measuring the volume delivered per unit of time. The flow rate was found to be directly proportional to the per cent stroke length and pressure (Fig. 3). Since the flow rate has been shown to be inversely proportional to the temperature (97), the column was maintained at constant temperature, 45°C, through the use of a constant temperature circulating bath.

The flow rate used in this investigation was 22.8 ml/hr for 9 hours and was then switched to 70.8 ml/hr for the remainder of the analysis.

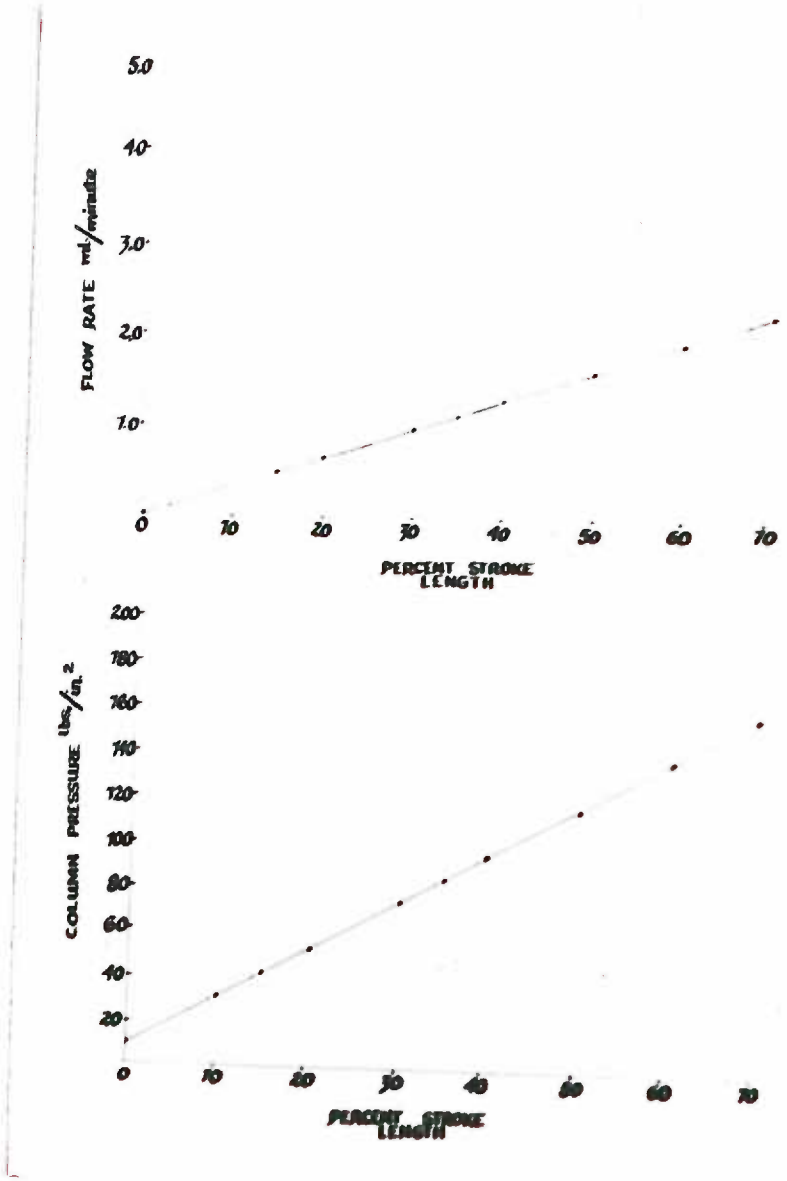
(e) Calibration of wavelength settings of the Beckman DB spectrophotometer. Small changes in the wavelength settings produce large changes in the absorbance measurements when the wavelength used is not close to the absorbance maximum. To calibrate the 260 and 280 mμ positions, one solution having a rapid change in absorbance with change in wavelength at 260 mμ and another solution having similar characteristics at 280 mμ were used. The absorbance of these solutions was determined at the band width used in the recording system. In this investigation the spectrophotometer slit is set at 1.0 mm (manual setting). The standards used in calibration were: (1) 0.1 N HCl and

(Figure 2). The 600 ml Pyrex gradient cylinder which are connected in series. A magnetic stirrer and heat lamp is associated with the first cylinder.



(Figure 3). The upper graph shows the linear relationship of per cent stroke length of the buffer pump to column flow rate.

The lower graph indicates a linear relationship of per cent stroke length of the buffer pump to column pressure.


5.0
4.0
3.0
2.0
1.0
FLOW RATE ml/minute
0
10
20
30
40
50
60
70
PERCENT STROKE LENGTH
200
180
160
140
120
100
80
60
40
20
COLUMN PRESSURE lbs/in.²
0
10
20
30
40
50
60
70
PERCENT STROKE LENGTH

(2) cytosine (10 µg/ml) in 0.1 N HCl and (3) adenine (10 µg/ml) in 0.1 N HCl. To adjust the spectrophotometer, the solution (2) was read against (1) and the 260 mµ setting adjusted until maximum absorption was obtained; similarly, solution (3) was read against (1) and the 280 mµ setting adjusted.

(f) Regeneration of column. To regenerate the resin, the column was unpacked by clamping it in an inverted position over a large beaker and then washing the resin out with glass distilled water by running PE 190 polyethylene tubing up the column. The resin was regenerated by the procedure described in part (b) of this section and repacked into the column.

Assay procedure

Following the preparation of urine for assay and determining the O.D.U./ml of the $HClO_4$-treated urine, 168 O.D.U. of the sample was placed into the column with a disposable tuberculin syringe. The sample was put onto the resin bed with air pressure at approximately 40 lbs/in^2. After the material was placed on the column, the column was filled with 0.15 M acetic acid-sodium acetate buffer, pH 4.4. The column plunger was put into place and the pump set to deliver a flow rate of 0.38 ml/min and the fraction collector set to collect 2.5 ml fractions. At the end of 9 hours the flow rate was changed to 1.18 ml/min and the fraction collector re-set to collect 5.0 ml fractions. The convex gradient was pumped through the column until 150 ml of buffer was left. At this time an additional 1200 ml of 3.0 M acetic acid-sodium acetate buffer was added. The chromatographic separation was continued until 2000 ml had been pumped through the column. The collected fractions were placed in a refrigerator until the chromatographic assay was finished, usually for about 40-48 hours, for purpose of further analysis. The

fractions containing the separated peaks were stored in 5 oz Wheaton bottles at 0-5°C.

Preparation of fractions for characterization

Fractions containing the separated peaks were pooled from five experiments. This was necessary to ensure adequate amounts of isolated material for analysis. The sample was placed in a lyophilization flask and "shelled" in the flask by rapidly rotating the flask, tipped to approximately an 80° angle, in a mixture of dry ice and acetone. After the solution had frozen as a "shell" within the flask, the flask was hooked immediately to an all glass lyophilizer and the vacuum started. The material was lyophilized for approximately 12 hours depending upon the starting volume. Upon completion of the lyophilization, the material was scraped from the vessel wall with a clean stainless steel spatula and transferred to a screw cap bottle, sealed tightly, and stored at 0-5°C until the desalting procedure. Any residue left in the flask was washed off with 1-2 ml of glass distilled water, put into a 10 dram vial,and placed into a vacuum oven at room temperature under 27-30-in Hg vacuum. The vacuum oven contained phosphorus pentoxide and sodium hydroxide as absorbing agents.

Desalting

(a) Preparation of activated charcoal. To prepare the charcoal, 60 g Norit A (Merck) was refluxed for 3-4 hours in 260 ml 2 M HCl. The refluxed Norit was then filtered on a Buchner funnel lined with two layers of filter paper (Whatman No. 30) and resuspended in 260 ml 2 M HCl. The suspension was again refluxed for 3-4 hours. This procedure was repeated three times. After the final refluxing, the Norit was filtered on a Buchner funnel and the suspension washed with glass distilled water until neutral. The neutral suspension was then washed

with two portions of 100 ml 95% ethanol/H_2O/conc. NH_3 (50:45:5) solution. The Norit was again washed with glass distilled water until neutral and sucked dry. The dry Norit was transferred to a bottle and suspended in 500 ml of glass distilled water to make a suspension containing approximately 100 mg Norit/ml. The activated charcoal suspension was stored at room temperature.

(b) Preparation of column. To make the desalting column, a small amount of Pyrex glass wool was packed into the bottom of a 5 3/4-in Pasteur pipet (Kimble Products, Cat. No. 72020). A slurry of thin layer chromatography cellulose MN 300 (Beckman Instrument, Inc.) was then poured into the pipet to a depth of 3-5 mm. The column was subsequently packed with 1 ml of well-suspended activated charcoal (approximately 100 mg of charcoal). The packed column was washed with 0.01 N HCl and the effluent checked at 260 mμ until stabilized. The column was then considered ready for the sample.

(c) Desalting procedure. The lyophilized fraction was dissolved in a minimum volume of glass distilled water. The material was placed on the column and passed through by 3 lbs/in^2 air pressure. The salt was allowed to pass through the column and the material remained on the activated charcoal bed. The column was then eluted with a solution of 95% ethanol/H_2O/conc. NH_3 (50:45:5). The effluent was checked at 260 mμ until the compound had been eluted. Generally, 25 ml of eluant was sufficient in eluting the material from the column. The eluted sample was placed in a 10 dram screw cap vial and placed in a vacuum oven containing sodium hydroxide pellets and phosphorus pentoxide at room temperature until dry.

Standards[2]

It was convenient to make up 10 mM single solutions of each

compound, with the exception of guanine which was made up to 1.0 mM. After the compound was weighed and placed in 10 ml volumetric flasks, the compound was treated with 0.5 N $HClO_4$, neutralized with 5.0 N KOH, the pH adjusted to 5.8, and finally diluted to volume. From these stock solutions the following standards were prepared to be 2 mM with respect to each compound, except guanine which was present in a final concentration of 0.2 mM and 5-methyl-CMP in a final concentration of 0.5 mM (Table 1). The standard mixtures were divided into 2 ml aliquots and stored frozen in 3 dram screw cap vials.

The standards were placed on the column and separated under the identical conditions as were the urine samples. Their retention volume and elution position were noted and the base ratios calculated. From these two parameters tentative identification of the eluted peaks in the recorded chromatography profiles could be made.

The commercial source for the compounds used as standards were as follows: California Biochemical Research- Cyt, Cyd, Urd, Ura, Thy, Ino, Ado, Ade, Guo, Gua, CMP, UMP, AMP, GMP, dCyd, dAdo, dGuo, Thd, dCMP, dAMP; Sigma Chemical Company- IMP, CDP, UDP, ADP, GDP, CTP, UTP, ITP, ATP, GTP, dGMP, dTMP, 5-methyl-dCMP.

[2] Abbreviations in this thesis are based on accepted usage of the Journal of Biological Chemistry, as follows:

Bases: Cyt, Ura, Thy, Ade, Gua.
Ribosides: Cyd, Urd, Ino, Ado, Guo.
Ribonucleotide monophosphates: 5'-CMP, -UMP, -IMP, -AMP, -GMP.
Ribonucleotide diphosphates: CDP, UDP, IDP, ADP, GDP.
Ribonucleotide triphosphates: CTP,UTP, ITP, ATP, GTP.
Deoxyribosides: dCyd, dAdo, dGuo, Thd.
Deoxyribonucleotides: dCMP, dTMP, dGMP, 5-methyl-dCMP.

TABLE 1.

Standard Mixtures

Standard	Mixture
A	Cyt, Cyd, Urd, Ura, Thy
B	Ino, Ado, Ade, Guo, Gua (0.2 mM)
C	CMP, UMP, IMP, AMP, GMP
D	dCyd, dAdo, dGuo, Thd
E	dCMP, dTMP, dAMP, dGMP+5-methyl-dCMP (0.5 mM)
F	Cyt, Thy, Ade, Gua (0.2 mM)
G	CDP, UDP, ADP, GDP
H	CTP, UTP, ITP, ATP, GTP

RESULTS

Nucleated cell counts

In the infected groups there were marked differences in the nucleated cell counts from those observed in the controls (Fig. 4). The results indicate a temporal progressive increase in the total nucleated cell counts in the infected groups. Group A animals showed a rapid rise in total nucleated cell counts between the second and eighth weeks, which was apparently due to the high susceptibility of these animals to the virus as reflected in their death rates (Table 2). A slight lag in the increase in total nucleated cells during this same period occurred in the group B animals. The maximum group B total nucleated cell counts were attained by the ninth week, and were not as high as those observed in group A animals. It was further observed that group B animals had a drop in nucleated cell counts between the tenth and fifteenth weeks. This decrease and the observed lag in increase of total nucleated cell count was probably due to data obtained from animals which were more resistant to the leukemogenic virus. As a result, some of these mice survived until week nineteen. The control group showed only a slight variation in the total nucleated cell count throughout the course of the experiment. The graphic representation of the total nucleated counts reflected the temporal changes which had occurred in lymphocytes, polymorphonuclear leukocytes, and erythroblasts (Fig. 5, 6, and 7).

The maximum lymphocyte count occurred during the fifth week in group A animals. Group B animals showed an apparent lag in the lymphocytic response and did not attain a maximum until week nine. It was further noted that there was a drop in lymphocyte count following maximum activity which occurred in group A animals between weeks five and

eight and between weeks nine and ten in group B animals. The group B data, following the initial drop in activity, suggested a slow temporal increase in lymphocyte counts. Another interesting observation was that group B animals indicated a more pronounced lymphocytic response than did group A animals.

The appearance of erythroblasts in group A occurred during week two and attained a maximum by the eighth week. Group B animals also showed the appearance of these cells by the second week but did not reach their maximum activity until week nine. Both groups showed a decrease in erythroblasts following the maximum activity. It was further observed that the group A animals elicited a more pronounced erythroblastic response than did group B.

The polymorphonuclear counts did not show any appreciable difference between the two infected groups. Elevation of these counts were observed to occur between the second and ninth weeks. The maximum counts seen in group B animals at week nine were followed by a decrease in counts from week ten through week sixteen.

The lymphocyte, erythroblast, and polymorphonuclear counts were observed to be markedly higher in the infected groups, A and B, than those of the control, Group C, animals.

Chromatographic elution profiles of urine

Serial samples of control and infected urine were analyzed in identical fashion to determine if changes in elution profiles could be correlated with the pathogenesis of the disease. Representative chromatographic profiles showed remarkable similarity in their eluted patterns, except for peaks 13 and 21 (Fig. 8-25). The appearance of peak 13 and its progressive increase and decrease correlated well with the concomitant increase and decrease of total nucleated cell counts (Tables 7 and 8)

(Figure 4). Graphic representation of the average total nucleated cells per cubic millimeter tabulated from Tables 3, 4, and 5 in control and infected animal groups. The graph reflects the pathogenic development of the Rauscher virus infection.

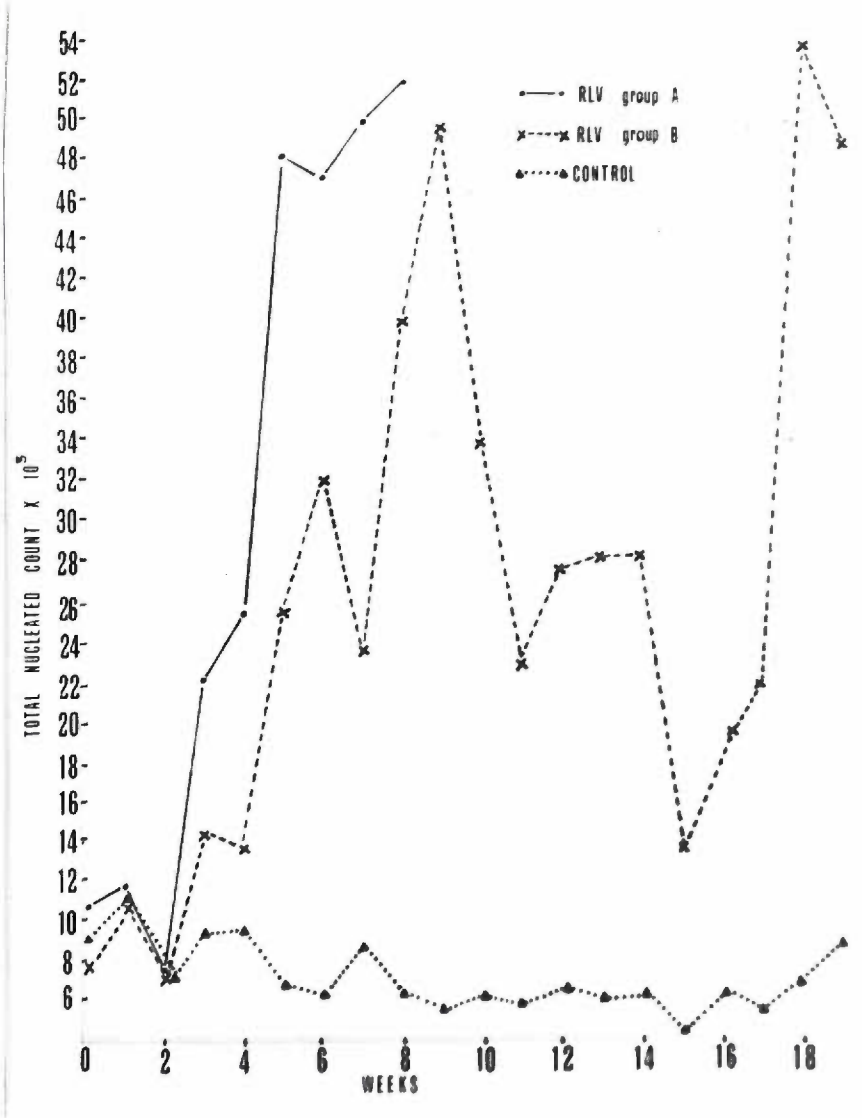
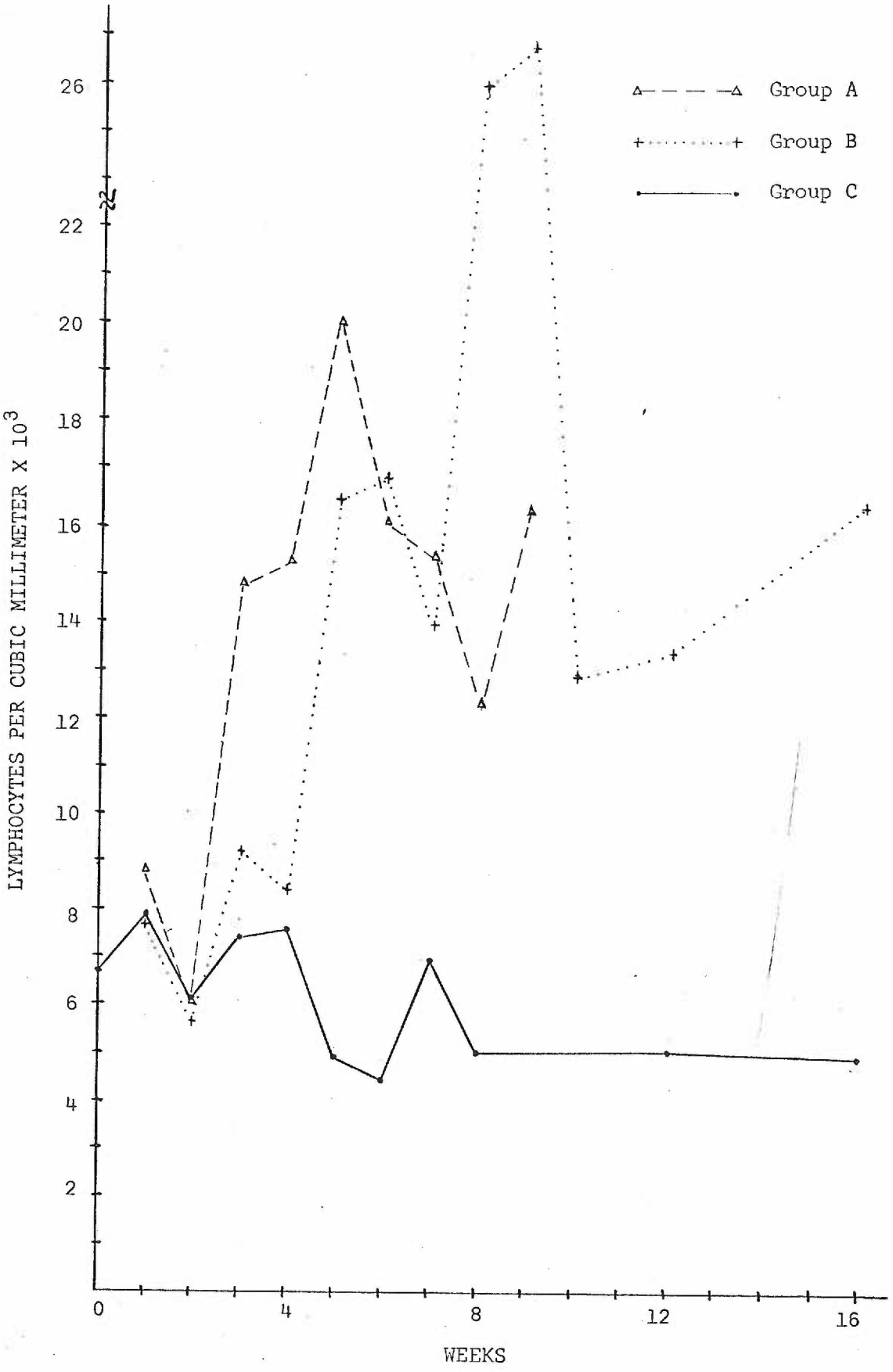

RLV group A
RLV group B
CONTROL
TOTAL NUCLEATED COUNT X 10^5
WEEKS
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
0
2
4
6
8
10
12
14
16
18

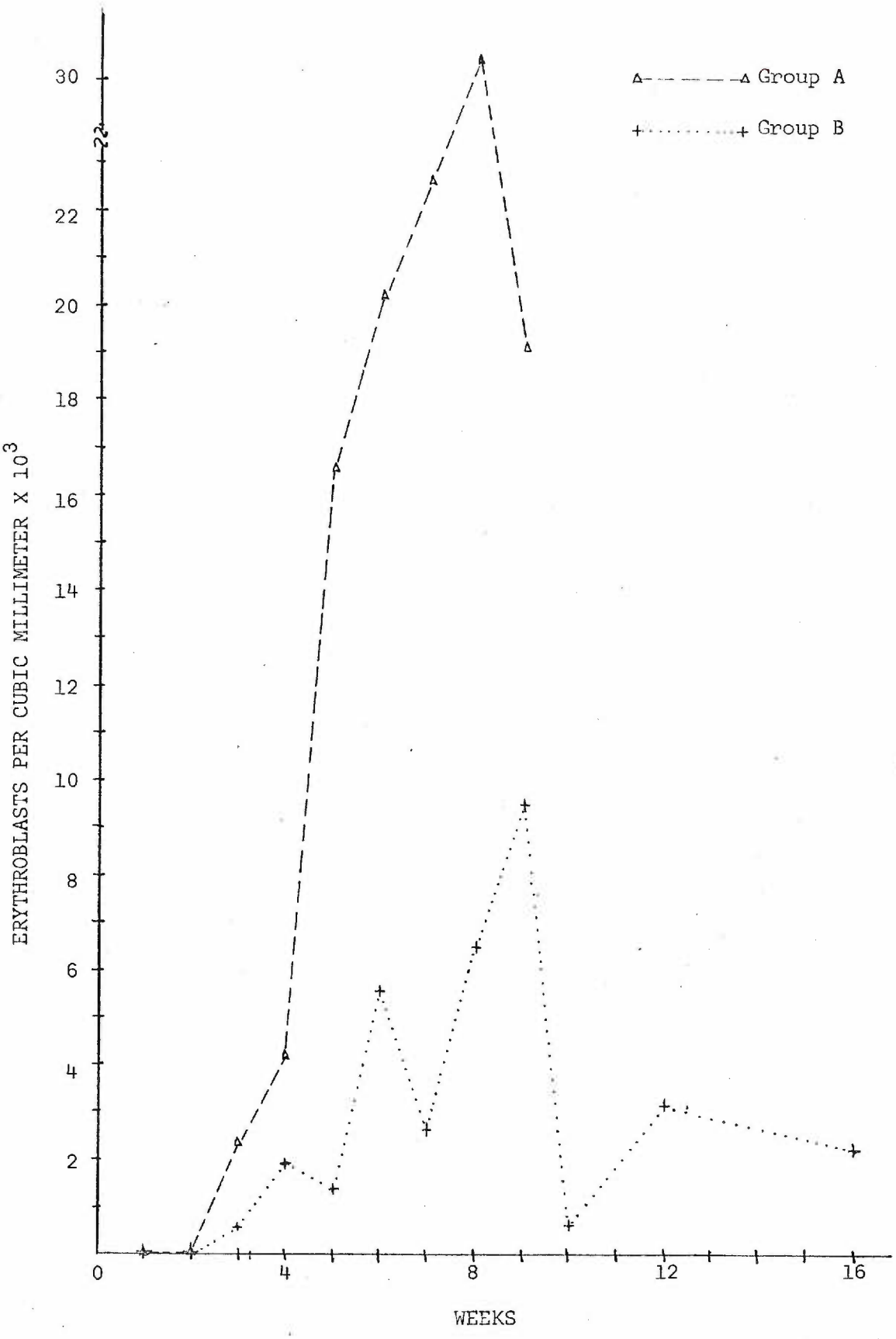
TABLE 2
ANIMAL DEATH RECORD

		WEEK																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
NUMBER OF LIVING ANIMALS	CONTROL	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	GROUP A INFECTED	8	8	8	8	4	3	2	1												
	GROUP B INFECTED	8	8	8	8	8	8	6	4	4	3	3	3	3	3	3	3	3	3	3	2

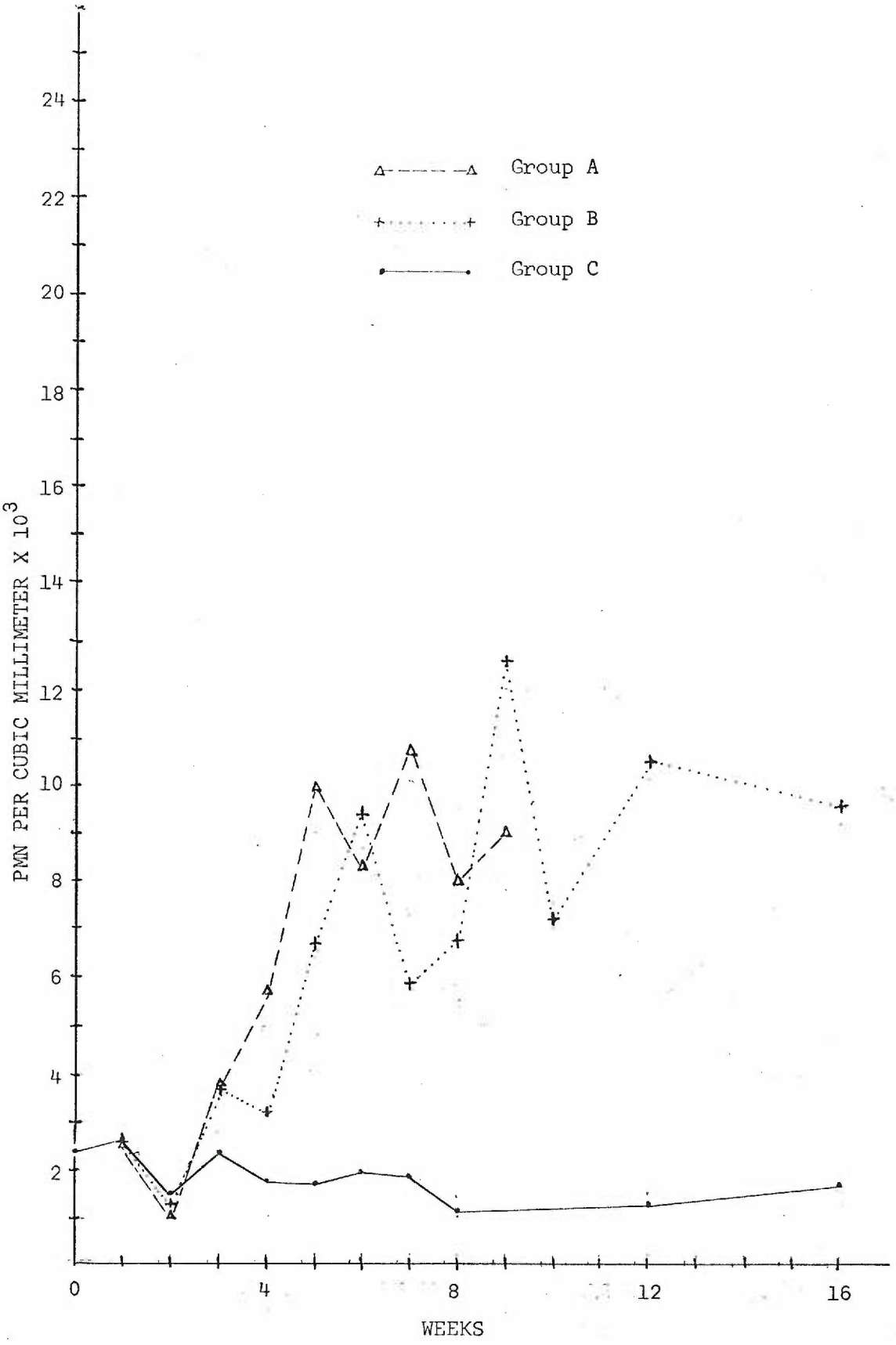
(Figure 5). Shown is a graph of the average total lymphocyte counts per cubic millimeter which contributes to the total nucleated cell count data of control and infected groups. This data was tabulated from Tables 18, 19, and 20.


Group A
Group B
Group C
LYMPHOCYTES PER CUBIC MILLIMETER X 10^3
26
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
WEEKS

(Figure 6). Shown is a graph of the average total erythroblast counts per cubic millimeter tabulated from Tables 18, 19, and 20 which contributes to the total nucleated cell data of control and infected groups.


Group A
Group B
ERYTHROBLASTS PER CUBIC MILLIMETER X 10^3
30
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
WEEKS

(Figure 7). A graphic representation of the average total polymorphonuclear leukocyte counts per cubic millimeter tabulated from Tables 18, 19, and 20 which contributes to the total nucleated cell data of control and infected groups.


Group A
Group B
Group C
PMN PER CUBIC MILLIMETER X 10^3
24
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
WEEKS

observed in the infected animals. There was an occasional indication of some slight amounts of peak 13 material in the uninfected controls (Table 6) which may have been due to data obtained from the control group having a periodic high nucleated count.

Peak 21 was normally present in the control animals (Table 9). However, it was also observed to increase and decrease in direct reflection of the total nucleated cell counts of the infected groups (Tables 10 and 11).

Peak 16 was considered since it appeared that this sharply eluting peak remained relatively constant throughout the course of the experiment, and did not appear to reflect the pathogenesis of the disease (Tables 12, 13, and 14) and could, therefore, be used to correct for differences in O.D.U. of urine placed on the column. A statistical treatment of the areas under peak 16 obtained from control and infected groups was considered to determine if the area was significantly different or if the areas were similar. An IBM 1130 computer programmed to perform an analysis of variance showed the variance, when an F-ratio of 9.355 was chosen, to be very significant. The control group had a F-ratio of 6.70 with respect to infected group A, and was probably significantly different. An F-ratio of 17.38 was obtained when controls were compared with infected group B which constituted a very significant difference. Groups A and B were shown not to be significantly different from each other. Consequently, the data was tabulated in terms of a standard O.D.U. of 168.

When a ratio of peak 13 to peak 16 was considered, a very good correlation was obtained between the ratio value and the total nucleated counts observed in control and infected groups. The temporal consideration of the peak ratio values showed that group A ratios

were greater and increased more rapidly than those ratios obtained from group B. This was consistent with the total nucleated cell count data. A similar observation was made when the ratio of peak 21 to peak 16 was determined (Tables 15, 16, and 17).

Neither peaks 13 or 21, nor their ratios to peak 16, could be directly correlated with any specific group of cells, lymphocytes, erythroblasts, polymorphonuclear leukocytes, which contributed to the total nucleated cell data. A correlation of this nature was obscured by virtue of the experimental design of this investigation, that is, the chromatographic elution profiles were from pooled urines obtained from animals which may have been in different pathological states of the disease. Since peaks 13 and 21 appeared to reflect the pathogenesis of the virus infection, as evidenced by their close correlation to the total nucleated cell counts, characterization of these peaks were subsequently attempted.

Characteristics of peak 16 (98)

After the peak 16 samples had been pooled, lyophilized, and desalted, an absorption spectrum was determined on the sample with a Carey, Model 15, spectrophotometer in attempts to obtain some identifying characteristics. A spectrum was obtained (Fig. 26) where the maximum absorption was at 262 mμ, which indicated the presence of some ultra-violet absorbing material. The difference between the maximum and the minimum, at 240 mμ, was not great indicating that further desalting and purification was necessary. The sample was then desalted on activated charcoal three times. The sample was streaked on paper for paper chromatography separation, using a butanol/water solvent. The results of this separation (Fig. 27) showed the presence of two bands, "a" and "b". Samples "a" and "b" were cut out and eluted with

glass distilled water and their respective absorption spectra determined.

Band "b" had an absorption maximum at 268 mμ with a minimum at 235 mμ at pH 2.0, while a spectrum under alkaline conditions indicated a spectral shift with a maximum at 280 mμ and a minimum at 240 mμ (Fig. 28). The peak was identified as xanthine.

Band "a" had a maximum at 266 mμ and a minimum at 212 mμ under acid conditions, pH 2.0; while under alkaline conditions, pH 12.0, a spectral shift occurred to give a new maximum at 278 mμ and a new minimum at 236 mμ (Fig. 29). This compound has not been identified.

Characteristics of peak 13 (98)

Following the activated charcoal desalting procedure, a spectrum was determined on the sample. When the sample was taken up in glass distilled water, no distinguishable spectrum was obtained and appeared to be principally salt. A slight shoulder appeared at the 305 and the 265-270 mμ range when the sample was acidified to pH 2.0. Under alkaline conditions, pH 12.0, a very slight spectral shift was observed with shoulders appearing in the regions of 310-315 mμ and 270-275 mμ. The sample did not appear to be soluble in water, but subsequently was found to be very soluble in 95% ethanol and sparingly soluble in chloroform. A spectrum of the sample was determined in 95% ethanol using an ethanol blank (Fig. 30) which shows an absorption maximum at 285 mμ and a minimum at 255 mμ. Since the sample was not soluble in water and no distinguishable spectrum was obtained, it was presumed not to be of nucleic acid nature. Because of the absorption at 285 mμ in 95% ethanol, the sample was checked to see if it was of a protein nature. Negative results were obtained when the sample was spotted on paper and sprayed with ninhydrin.

An infra-red spectroscopic analysis was performed using the KBr pellet technique. The infra-red spectrogram (Fig. 31) indicated that the sample was relatively simple in structure. The possibility that it was of aromatic nature may be ruled out due to the lack of a prominent peak in the 750-900 frequency (cm^{-1}) region. A possible O-H stretch of a hydroxyl group may be present as indicated by the peak at 3400. There was an indication of N-H stretching of an amine group as was shown by the very prominent peak at 3150. Small peaks were observed at 2960 and 2626 which are characteristic of some symmetric C-H stretch of a methyl group and an asymmetric vibration of an H atom in a methylene group. Stretching of C=O, such as, aldehydes, ketones, or esters was shown by the appearance of a peak at 1720. A possible amide group may contribute to the peak observed at 1630. The sharply rising peak at 1400 indicated a very strong C-H stretch. There appeared to be some part of an ester with a C-O stretch as was evidenced by peaks in the 1100-1250 region. The actual structure of the compound could not be derived from the infra-red data but suggested that it was a relatively simple structure and could possibly be a degraded amino acid. The data obtained does not rule out, however, the possibility that this could be a component derived from some other class of compound.

Characteristic of peak 21 (98)

No spectral peaks were obtained when the sample was dissolved in water. In 95% ethanol there appeared two shoulders, one at the 270-280 mμ region and the other at the 220-230 mμ region, on a continuously rising peak. The poor resolution of the two peaks may have been due to high salt concentrations still present in the sample or failure to recover the majority of the material from the column. Since the sample in ethanol and water solvents failed to have appreciable absorbance,

the activated charcoal column used originally to desalt the sample was further eluted with N-propanol/N-butanol/conc. NH_3 (50:45:5). A spectrum of the eluted material indicated only a slight shoulder on the curve at 270-280 mμ. The effluents from the two elutions were pooled, then chromatographed on paper using a butanol/water solvent (Fig. 27). Since there was no evidence of migration of the ultraviolet absorbing material, a sample strip was taken and sprayed with ninhydrin. A positive reaction slightly above the origin was observed. Further identification of the compound has not been undertaken at this time.

TABLE 3

INDIVIDUAL NUCLEATED
CELL COUNT

CONTROL (GROUP C)

	ANIMAL NUMBER							
	1	2	3	4	5	6	7	8
WEEK								
0	9000	12400	9150	8700	8100	9350	7650	8150
1	12000	11800	10100	11400	10600	13250	8750	9050
2	5000	7350	8500	9000	7450	7300	8100	9800
3	9200	11950	10250	10850	10150	9150	4450	8950
4	11000	8850	10000	11000	10850	8450	7100	10000
5	6050	6050	7550	10450	6850	8500	6250	4350
6	6250	6250	6000	7150	5200	6300	7450	8100
7	13450	12050	17000	6350	6850	6200	5850	6800
8	6050	7150	5850	7100	6000	6450	6350	5450
9	6250	5750	5200	6550	5550	4550	6150	5150
10	6450	6050	7050	6000	6650	5050	6150	7800
11	7100	6300	6200	5650	6450	5100	6450	6100
12	7100	6450	6300	7100	6200	6100	6450	6650
13	10100	6550	7450	5450	5900	5150	7050	4600
14	10000	9450	5350	5950	5100	6100	7400	5100
15	*	8000	4000	5150	*	*	*	*

*= ANIMALS ALIVE, BUT INDIVIDUAL NUCLEATED CELL COUNTS WERE NOT MADE.

TABLE 3 (Continued)

INDIVIDUAL NUCLEATED
CELL COUNT

CONTROL (GROUP C)

	ANIMAL NUMBER							
	1	2	3	4	5	6	7	8
WEEK								
16	*	5650	5950	7550	*	*	*	*
17		4150	5600	6950				
18		7300	8400	4850				
19		8100	7500	11750				

*= ANIMALS ALIVE, BUT INDIVIDUAL NUCLEATED CELL COUNTS WERE NOT MADE.

TABLE 4

INDIVIDUAL NUCLEATED
CELL COUNT

GROUP A (INFECTED)

	ANIMAL NUMBER							
	1	2	3	4	5	6	7	8
WEEK								
0	10600	10900	11700	10000	10050	12350	8950	10650
1	9100	10350	15450	14200	11850	12150	10650	9800
2	12750	8000	5650	6550	5650	5300	8300	6150
3	24600	25650	40950	27100	14750	18200	12350	12650
4	26600	33500	28000	+	17700	27700	17300	26450
5	70000	+	65000		29800	+	27450	+
6	52400		+		44000		44650	
7	36500				+		63150	
8	51600						+	
9	+							

+= DEATH

TABLE 5

INDIVIDUAL NUCLEATED
CELL COUNT

GROUP B (INFECTED)

	ANIMAL NUMBER							
	1	2	3	4	5	6	7	8
WEEK								
0	10850	7150	6550	6000	8250	6550	8000	8400
1	9350	10050	12500	10500	10600	9750	9950	12150
2	7250	14950	6150	6100	5750	5850	5350	5000
3	19250	15250	16700	11650	17450	7100	8450	17500
4	18800	14800	20000	10000	10400	8650	9450	14850
5	74550	14250	22200	9900	23100	18000	18550	20400
6	40000	75000	33000	17450	+	6800	18500	+
7	26650	+	29000	27650		9000	+	
8	60800		50800	19850		27700		
9	112350		+	29250		6250		
10	43150			16450		40400		
11	41650			22250		3450		
12	50150			25200		6550		
13	46100			30800		10000		
14	47600			29300		10150		
15	21250			15950		3200		

+= DEATH

TABLE 5 (Continued)

INDIVIDUAL NUCLEATED
CELL COUNT

GROUP B (INFECTED)

	ANIMAL NUMBER							
	1	2	3	4	5	6	7	8
WEEK								
16	27200	+	+	78200	+	1950	+	+
17	31300			28300		5300		
18	52750			103600		3950		
19	88200			+		8850		

+= DEATH

TABLE 6

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

CONTROL - PEAK 13

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	204.0	1.7	.0083	1.4
2	168.0	0.8	.0048	0.8
3	168.7	0.7	.0041	0.7
4	166.4	0.0	.0000	0.0
5	169.6	0.4	.0024	0.4
6	165.0	*	-	-
7	169.8	0.1	.0006	0.1
8	166.7	1.5	.0090	1.5
9	167.0	0.0	.0000	0.0
10	168.0	0.0	.0000	0.0
11	168.0	0.0	.0000	0.0
12	168.5	*	-	-
13	167.9	0.5	.0030	0.5
14	166.6	1.0	.0060	1.0
15	168.0	0.0	.0000	0.0
16	167.9	0.0	.0000	0.0

* EXPERIMENT LOST

TABLE 7

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP A (INFECTED)
PEAK - 13

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	*	-	-	-
2	65.0	0.8	.0123	2.1
3	168.2	5.5	.0327	5.5
4	166.8	3.7	.0222	3.7
5	167.7	8.4	.0501	8.4
6	168.0	4.8	.0286	4.8
7	167.9	9.1	.0542	9.1

* EXPERIMENT LOST

TABLE 8

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP B (INFECTED)
PEAK - 13

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	168.7	0.0	.0000	0.0
2	167.3	3.5	.0209	3.5
3	167.9	5.9	.0351	5.9
4	167.3	1.9	.0113	1.9
5	169.1	9.1	.0538	9.0
6	165.1	3.4	.0206	3.5
7	167.3	4.3	.0257	4.3
8	167.5	2.6	.0155	2.6
9	*	-	-	-
10	168.7	1.7	.0101	1.7
11	167.8	2.5	.0149	2.5
12	168.1	3.2	.0190	3.2
13	150.7	2.4	.0159	2.7
14	168.0	1.4	.0083	1.4
15	167.6	3.4	.0203	3.4
16	167.7	1.3	.0078	1.3

* EXPERIMENT LOST

TABLE 9

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

CONTROL - PEAK 21

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	204.0	10.8	.053	8.9
2	168.0	9.4	.056	9.4
3	168.7	10.3	.061	10.2
4	166.4	3.7	.022	3.7
5	169.6	6.2	.037	6.2
6	165.0	2.0	.012	2.0
7	169.8	4.8	.028	4.7
8	166.7	3.7	.022	3.7
9	167.0	3.5	.021	3.5
10	168.0	6.0	.036	6.0
11	168.0	*	-	-
12	168.5	3.4	.020	3.4
13	167.9	6.2	.037	6.2
14	166.6	4.7	.028	4.7
15	168.0	8.0	.051	8.6
16	167.9	9.5	.057	9.6

* EXPERIMENT LOST

TABLE 10

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP A (INFECTED)
PEAK - 21

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	*	-	-	-
2	65.0	9.9	.1523	25.6
3	168.2	11.4	.0678	11.4
4	166.8	6.7	.0402	6.8
5	167.7	8.1	.0483	8.1
6	168.0	11.1	.0655	11.0
7	167.9	25.4	.1513	25.4

* EXPERIMENT LOST

TABLE 11

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP B (INFECTED)
PEAK - 21

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	168.7	13.1	.0777	13.1
2	167.3	9.6	.0574	9.6
3	167.9	9.5	.0566	9.5
4	167.3	6.5	.0389	6.5
5	169.1	10.9	.0645	10.8
6	165.1	10.0	.0606	10.2
7	167.3	9.4	.0562	9.4
8	167.5	18.7	.1116	18.7
9	*	-	-	-
10	168.7	10.1	.0599	10.1
11	167.8	16.1	.0959	16.1
12	168.1	10.7	.0637	10.7
13	150.7	14.9	.0989	16.6
14	168.0	9.5	.0565	9.5
15	167.6	11.8	.0704	11.8
16	167.7	8.0	.0477	8.0

* EXPERIMENT LOST

TABLE 12

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

CONTROL - PEAK 16

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	204.0	5.6	.0276	4.6
2	168.0	4.9	.0292	4.9
3	168.7	6.2	.0368	6.2
4	166.4	5.0	.0300	5.0
5	169.6	5.0	.0295	5.0
6	165.0	6.2	.0376	6.3
7	169.8	4.6	.0271	4.6
8	166.7	5.0	.0300	5.0
9	167.0	4.7	.0282	4.7
10	168.0	4.9	.0292	4.9
11	168.0	5.8	.0345	5.8
12	168.5	4.8	.0285	4.8
13	167.9	5.4	.0322	5.4
14	166.6	4.4	.0264	4.4
15	168.0	4.6	.0274	4.6
16	167.9	4.5	.0268	4.5

TABLE 13

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP A (INFECTED)
PEAK - 16

WEEK	O.D.U. URINE ON COLUMN	AREA $cm.^2$	$cm.^2$/O.D.U.	ADJUSTED AREA ($cm.^2$/O.D.U.) 168
1	*	-	-	-
2	65.0	2.5	.0385	6.5
3	168.0	5.3	.0315	5.3
4	166.8	6.3	.0378	6.4
5	167.7	5.5	.0328	5.5
6	168.0	6.8	.0405	6.8
7	167.9	5.6	.0334	5.6

* EXPERIMENT LOST

TABLE 14

TABULATION OF AREAS OBTAINED FROM RECORDED CHROMATOGRAPHIC PROFILES

GROUP A (INFECTED)
PEAK - 16

WEEK	O.D.U. URINE ON COLUMN	AREA	cm^2/O.D.U.	ADJUSTED AREA (cm^2/O.D.U.) 168
1	168.7	4.5	.0267	4.5
2	167.3	7.0	.0418	7.0
3	167.9	6.8	.0405	6.8
4	167.3	7.4	.0442	7.4
5	169.1	6.6	.0390	6.6
6	165.1	6.5	.0393	6.6
7	167.3	7.1	.0424	7.3
8	167.5	5.9	.0352	5.9
9	*	-	-	-
10	168.7	6.6	.0391	6.6
11	167.8	6.1	.0363	6.1
12	168.1	5.5	.0327	5.5
13	150.7	4.4	.0292	4.9
14	168.0	5.6	.0333	5.6
15	167.6	7.7	.0459	7.7
16	167.7	4.8	.0286	4.8

* EXPERIMENT LOST

TABLE 15

DETERMINATION OF AREA RATIOS OF
PEAK 16 TO PEAK 13 AND PEAK 21

CONTROL

WEEK	$\frac{\text{AREA (cm.}^2\text{) PEAK 13}}{\text{AREA (cm.}^2\text{) PEAK 16}}$	$\frac{\text{AREA (cm.}^2\text{) PEAK 21}}{\text{AREA (cm.}^2\text{) PEAK 16}}$
1	0.304	1.934
2	0.163	1.918
3	0.112	1.645
4	0.000	0.740
5	0.079	1.240
6	0.000	0.317
7	0.021	1.021
8	0.300	0.740
9	0.000	0.744
10	0.000	1.224
11	0.000	0.000
12	0.000	0.708
13	0.092	1.148
14	0.227	1.068
15	0.000	1.869
16	0.000	2.133

TABLE 16

DETERMINATION OF AREA RATIOS OF
PEAK 16 TO PEAK 13 AND PEAK 21

GROUP A (INFECTED)

WEEK	$\frac{\text{AREA (cm.}^2\text{) PEAK 13}}{\text{AREA (cm.}^2\text{) PEAK 16}}$	$\frac{\text{AREA (cm.}^2\text{) PEAK 21}}{\text{AREA (cm.}^2\text{) PEAK 16}}$
1	NO DATA	NO DATA
2	0.323	3.938
3	1.037	2.150
4	0.578	1.062
5	1.527	1.472
6	0.705	1.617
7	1.625	4.535

TABLE 17

DETERMINATION OF AREA RATIOS OF
PEAK 16 TO PEAK 13 AND PEAK 21

GROUP B (INFECTED)

WEEK	$\frac{\text{AREA (cm.}^2\text{) PEAK 13}}{\text{AREA (cm.}^2\text{) PEAK 16}}$	$\frac{\text{AREA (cm.}^2\text{) PEAK 21}}{\text{AREA (cm.}^2\text{) PEAK 16}}$
1	0.000	2.911
2	0.500	1.371
3	0.867	1.397
4	0.256	0.878
5	1.363	1.636
6	0.530	1.545
7	0.589	1.287
8	0.440	3.169
9	NO DATA	NO DATA
10	0.257	1.530
11	0.409	2.639
12	0.581	1.945
13	0.551	3.387
14	0.250	1.696
15	0.441	1.532
16	0.270	1.666

TABLE 18

AVERAGE NUMBER OF
NUCLEATED CELLS

GROUP C (CONTROL)

WEEK	NUMBER OF ANIMALS	TOTAL NUCLEATED CELLS	L/mm^3	PMN/mm^3	NUC. R./mm^3
0	8	9450	6700	2350	0
1	8	10850	7900	2600	0
2	8	7800	6100	1500	0
3	8	10100	7400	2300	0
4	8	9668	7590	1750	0
5	8	6800	4900	1700	0
6	8	6500	4450	1950	0
7	8	8800	6950	1850	0
8	8	6300	5000	1166	0
12	8	6543	5100	1243	0
16	8	6870	4964	1717	0

TABLE 19

AVERAGE NUMBER OF
NUCLEATED CELLS

GROUP A (INFECTED)

WEEK	NUMBER OF ANIMALS	TOTAL NUCLEATED CELLS	L/mm^3	PMN/mm^3	NUC. R./mm^3
1	8	11694	8805	2514	0
2	8	7419	6054	1105	0
3	8	21906	14852	3882	2388
4	7	25607	15211	5736	4200
5	4	48063	20090	9997	16582
6	3	47017	16127	8322	20217
7	2	49825	15446	10724	22670
8	1	51600	12384	8256	30444
9	1	46700	16345	9044	19040

TABLE 20

AVERAGE NUMBER OF
NUCLEATED CELLS

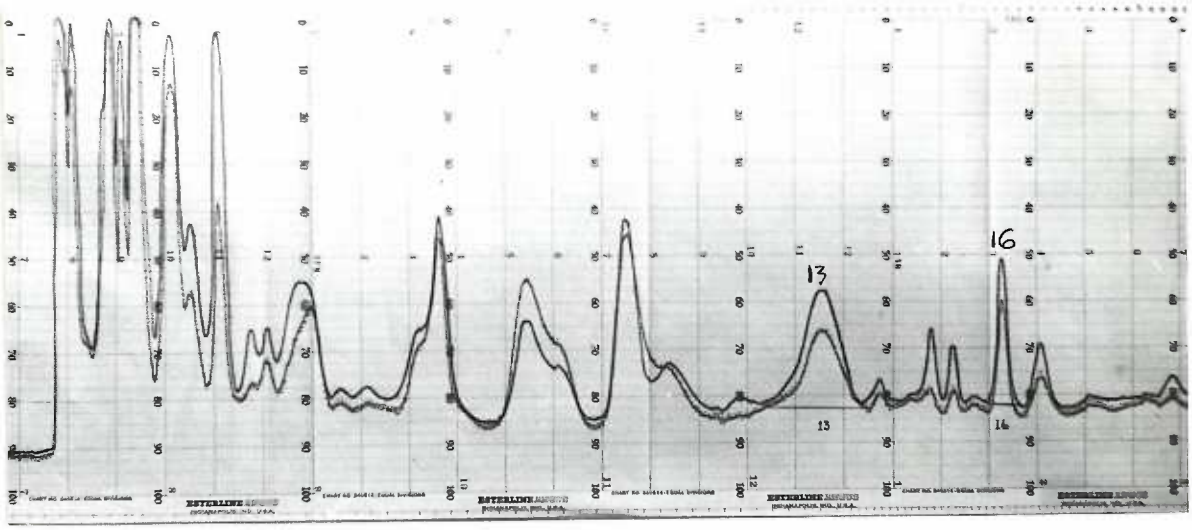
GROUP B (INFECTED)

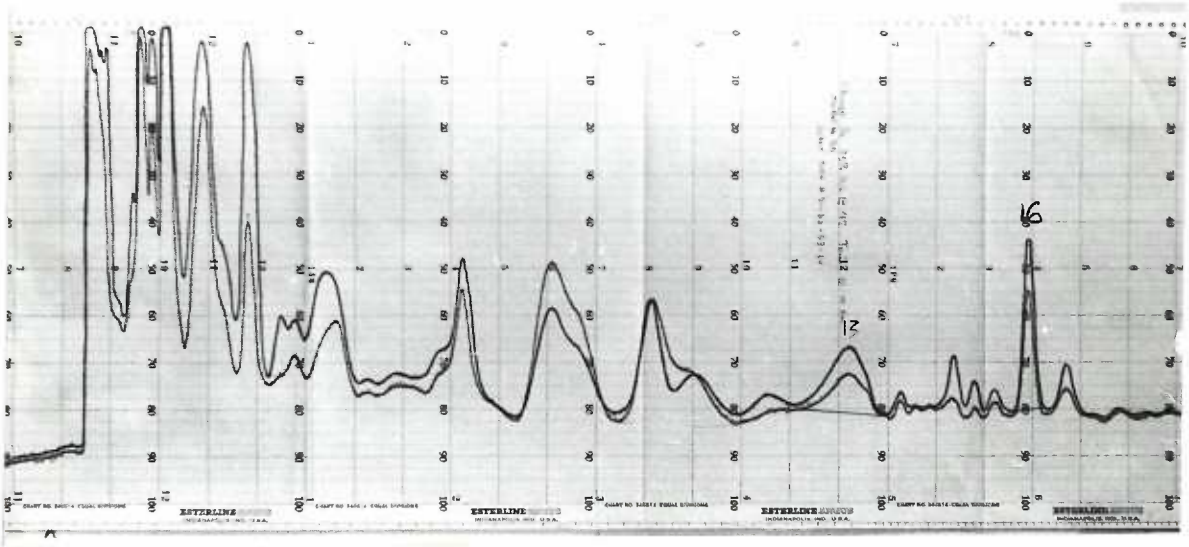
WEEK	NUMBER OF ANIMALS	TOTAL NUCLEATED CELLS	L/mm^3	PMN/mm^3	NUC. R./mm^3
1	8	10606	7647	2545	0
2	8	7050	5633	1234	0
3	8	14169	9201	3698	580
4	8	13370	8410	3195	1912
5	8	25119	16579	6682	1331
6	6	33460	16897	9436	5588
7	4	23075	13914	5884	2608
8	4	39788	25982	6764	6485
9	3	49283	26761	12606	9467
10	3	21333	12864	7189	575
12	3	27300	13377	10565	3085
16	3	29037	16464	9582	2323

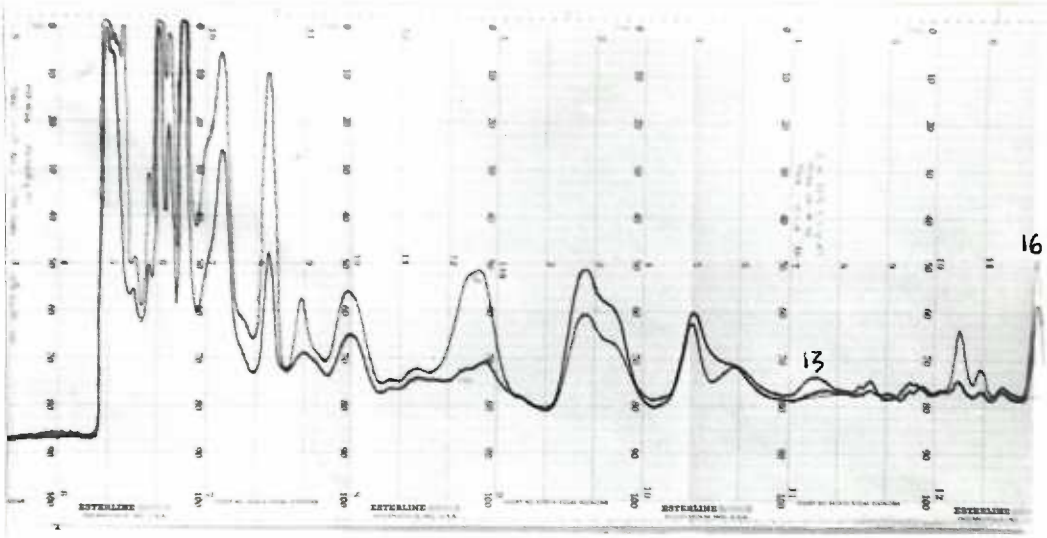
(Figure 8). A photograph of the recorded chromatographic elution profile of urine obtained from group A animals 5 weeks post-infection. Routinely, 168 O.D.U. of prepared urine was placed on the 0.9 X 115 cm Dowex 1-X8 column and eluted with a continuous convex gradient comprised of 600 ml 0.15 M acetic acid-sodium acetate buffer, 1200 ml 3.0 M acetic acid-sodium acetate buffer, both at pH 4.4. Flow rates were set at 22.8 ml/hr and 2.5 ml fractions collected for 9 hours at which time the flow rate was increased to 70.8 ml/hr and 5.0 ml fractions collected for the remainder of the analysis.

(Figure 9). A photograph of the recorded chromatographic elution profile of urine obtained from group B animals 5 weeks post-inoculation. Assay conditions were similar to those described in Figure 8.

(Figure 10). A photograph of the recorded chromatographic elution profile of urine obtained from group C, control, animals at 5 weeks under similar assay conditions. Note the remarkable similarity in profiles, except for peak 13 in groups A and B.


16
13
13
16

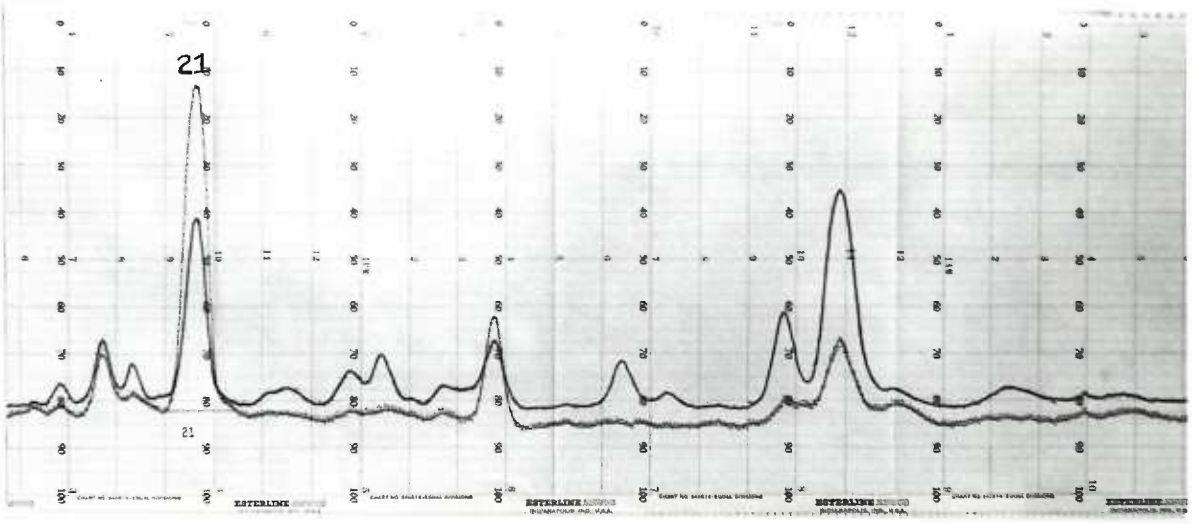

16
13


16
13

(Figure 11). This is a continuation of the profile seen in Figure 8. Of major interest is the increased elevation of peak 21.

(Figure 12). This is a continuation of the profile seen in Figure 9. Note the elevation of peak 21 with those observed in Figures 11 and 13.

(Figure 13). This is a continuation of the profile seen in Figure 10.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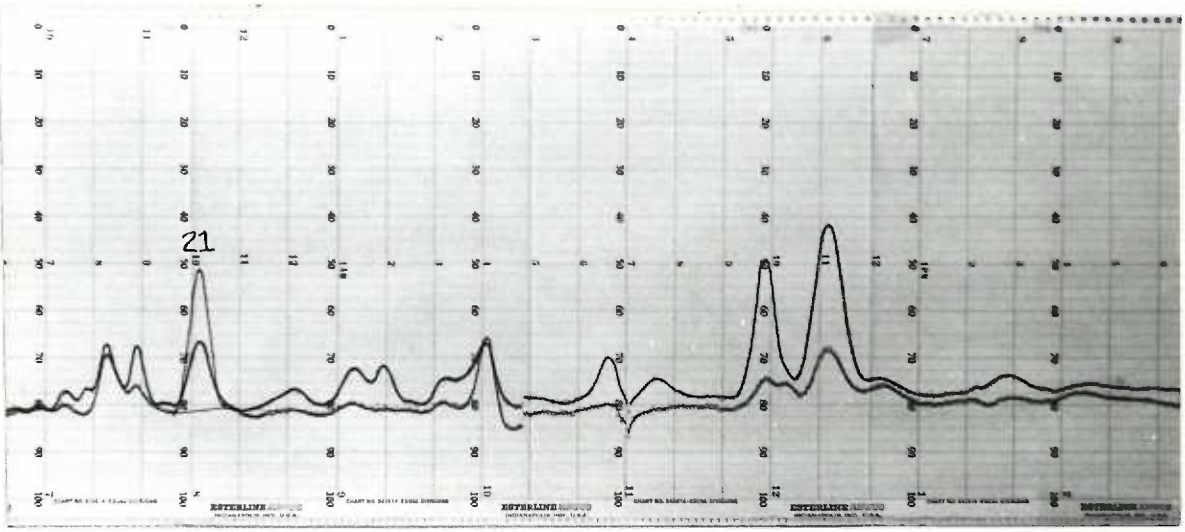
21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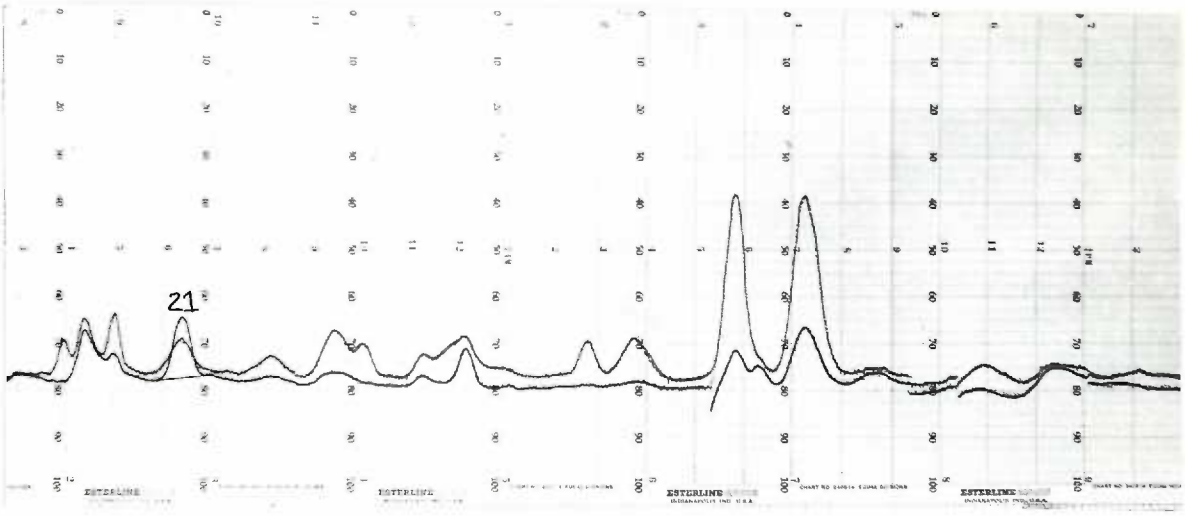
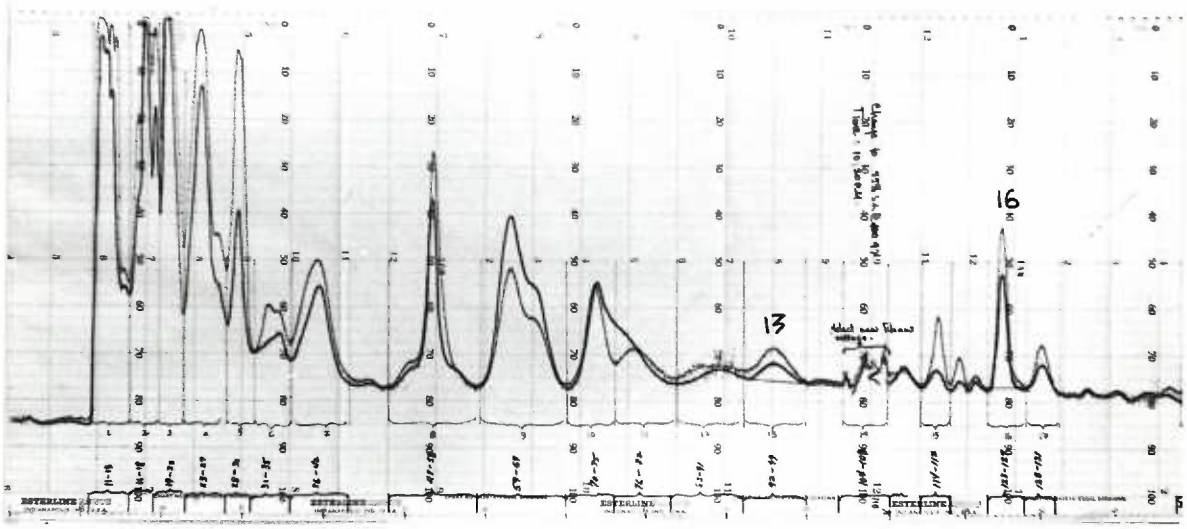
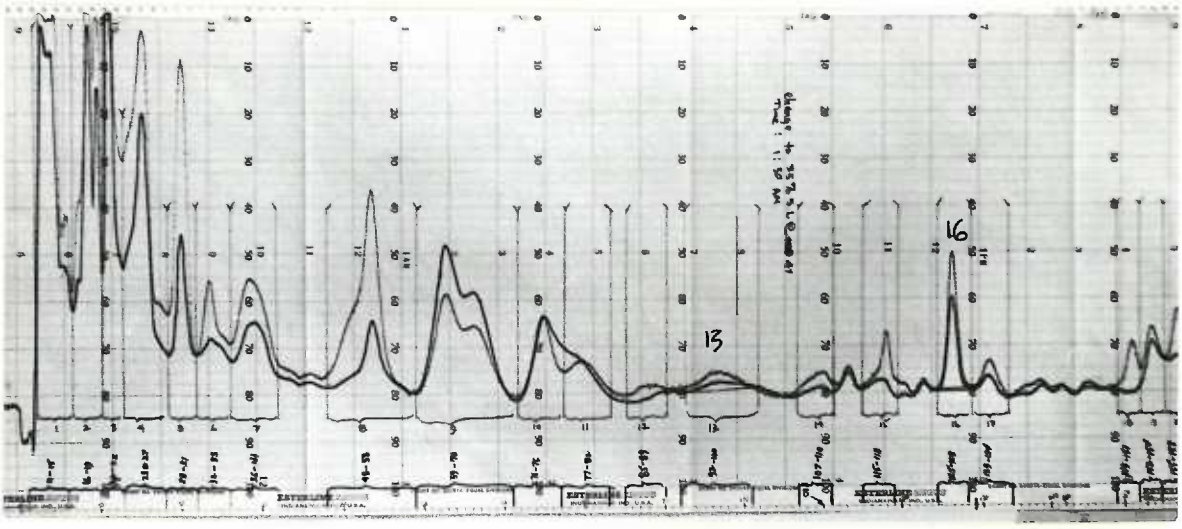

21

(Figure 14). A chromatographic elution profile obtained from group B animals 14 weeks post-infection. Peak 13 has decreased in direct correlation with the observed total nucleated cell count data.

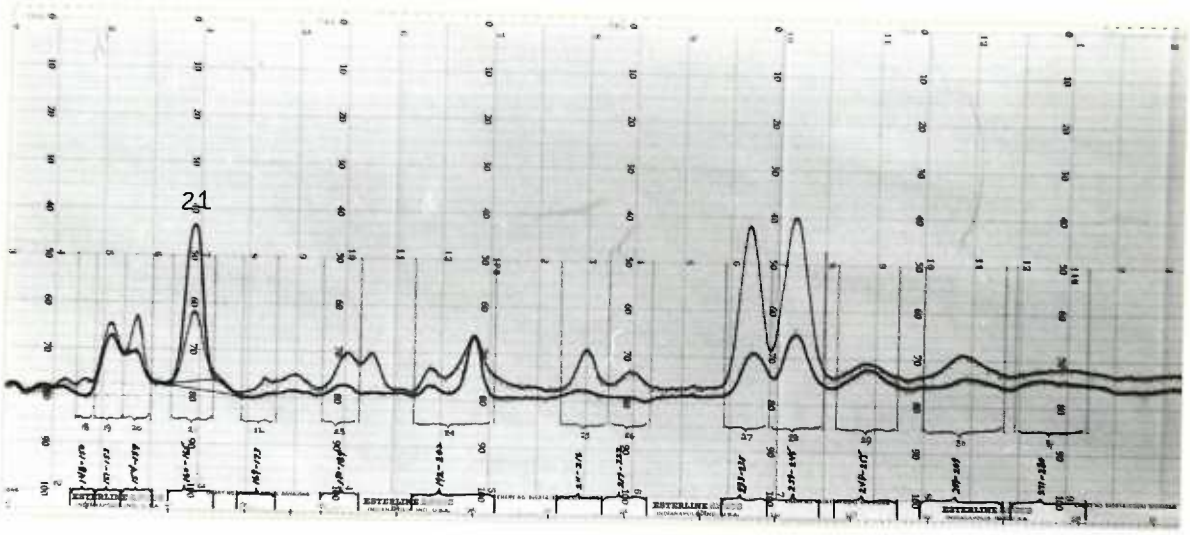
(Figure 15). A chromatographic elution profile obtained from group C, control, animals at week 14. There appears some peak 13 material but this observation was infrequent.


13
16


13
16

(Figure 16). This is a continuation of the profile seen in Figure 14. There still appeared an elevation in peak 21 which showed a temporal correlation to the total nucleated cell data.

(Figure 17). This is a continuation of the profile seen in Figure 15. Peak 21 is representative of those found in controls throughout this experiment.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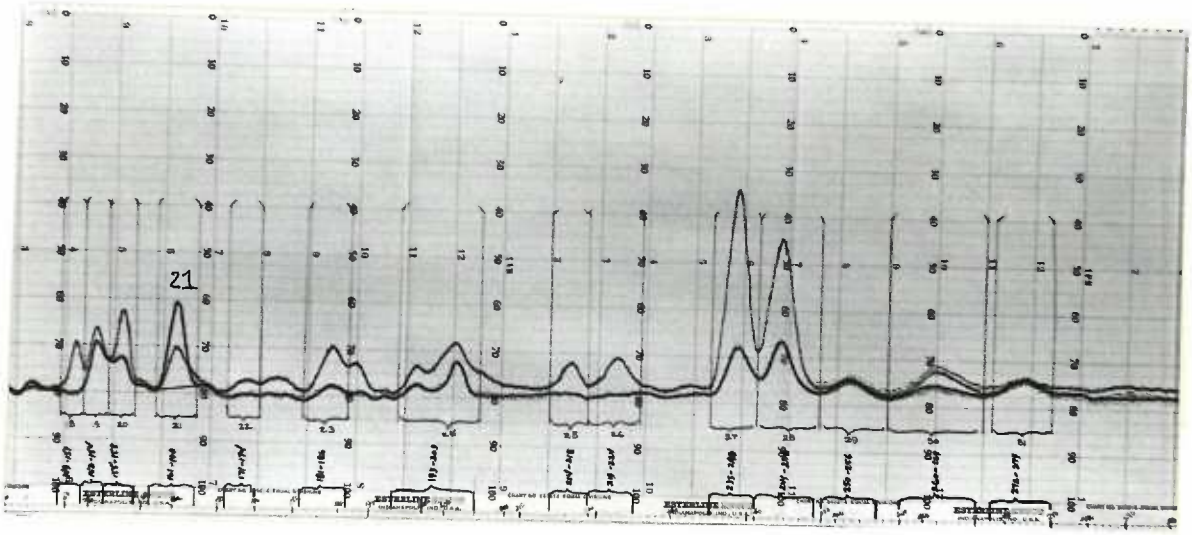
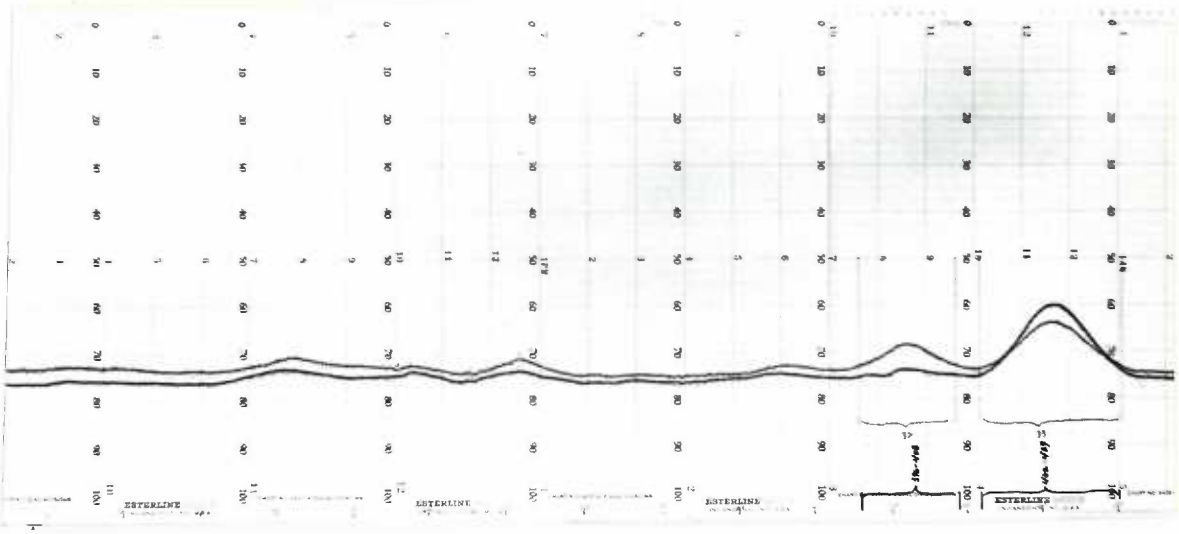
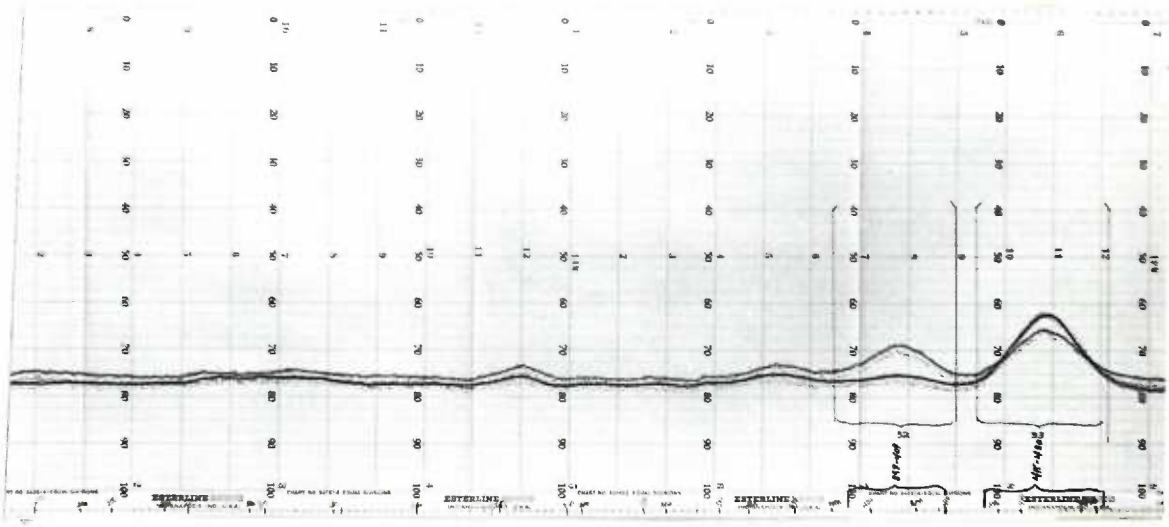

21

(Figure 18). A continuation of the profiles seen in Figures 14 and 16. The analysis was routinely eluted to this point in approximately 40 hours.

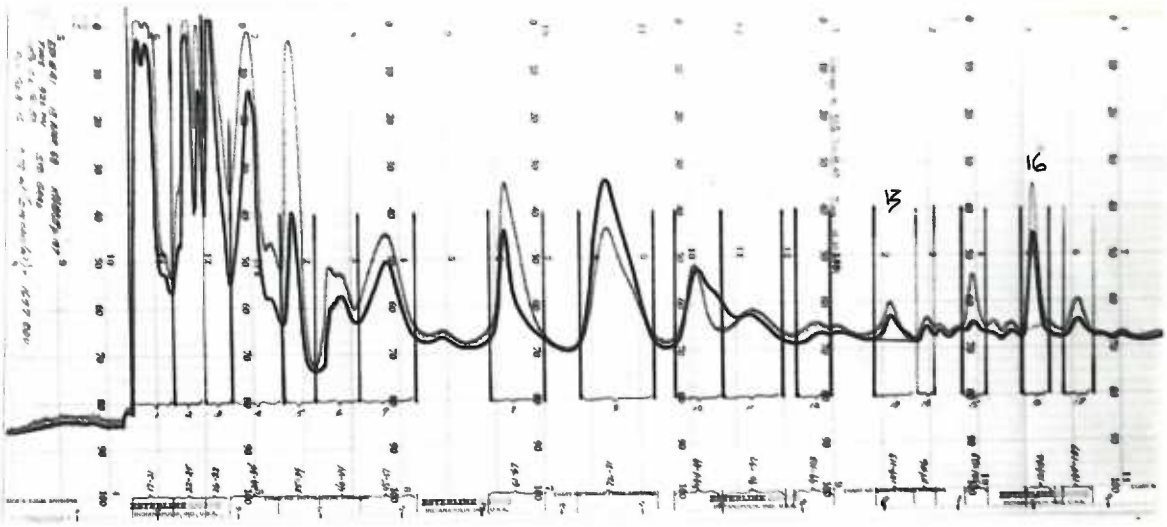
(Figure 19). A continuation of the profiles seen in Figures 15 and 17. Note the similarity of the profile with Figure 18.





(Figure 20). A chromatographic elution profile of group B animals 16 weeks post-inoculation. Peak 13 still correlates well with the total nucleated count data.

(Figure 21). A chromatographic elution profile of group C, control, animals, at 16 weeks. There may be some slight amounts of peak 13 material present.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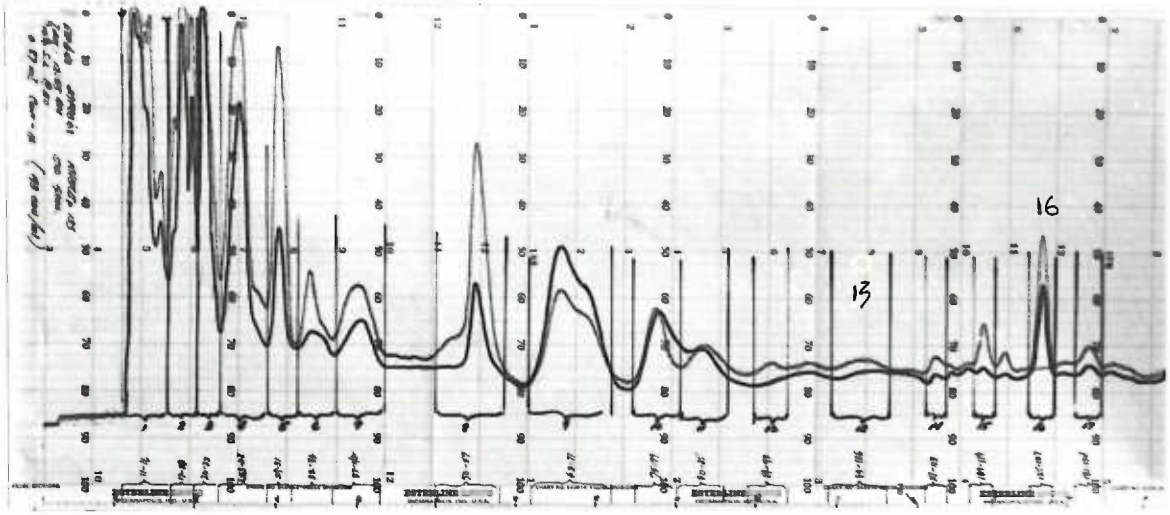
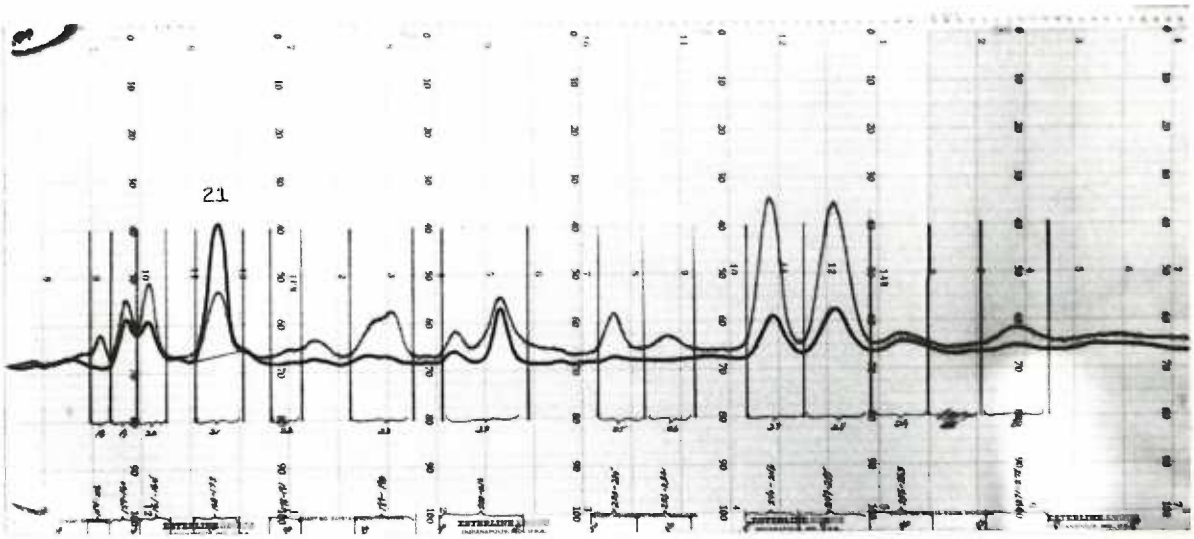
16


13
16

(Figure 22). A continuation of Figure 20. The activity of peak 21 was not markedly different from that observed in Figure 23.

(Figure 23). A continuation of Figure 21. The amount of peak 21 has increased from those observed in earlier controls.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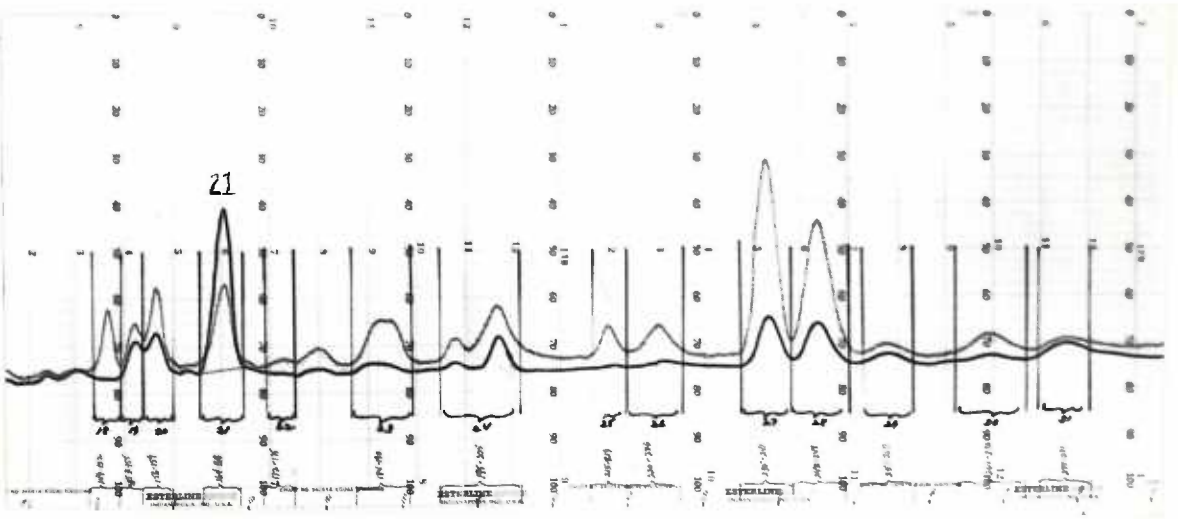
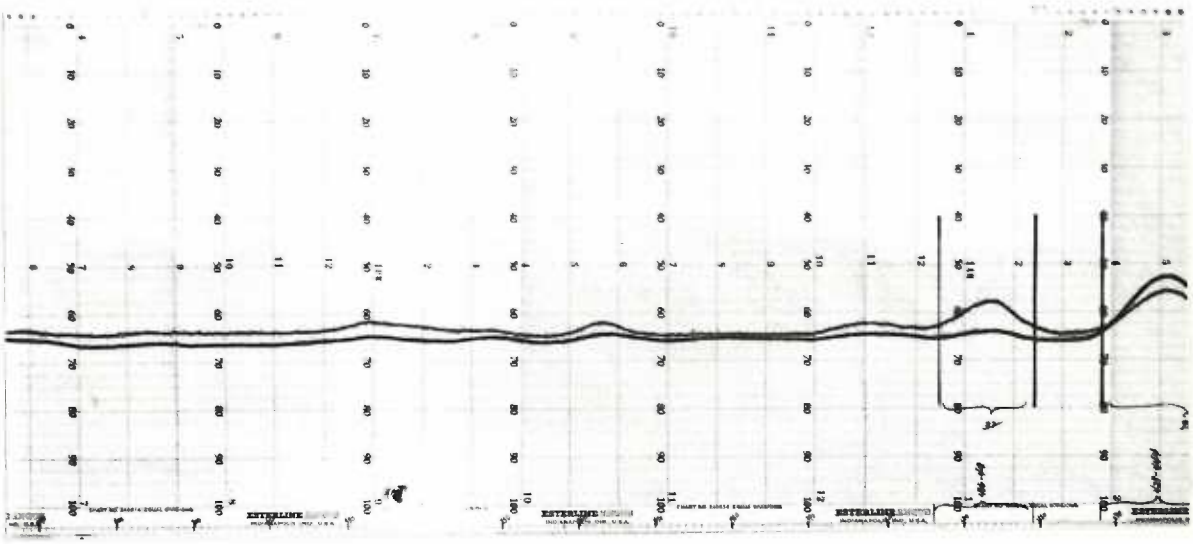
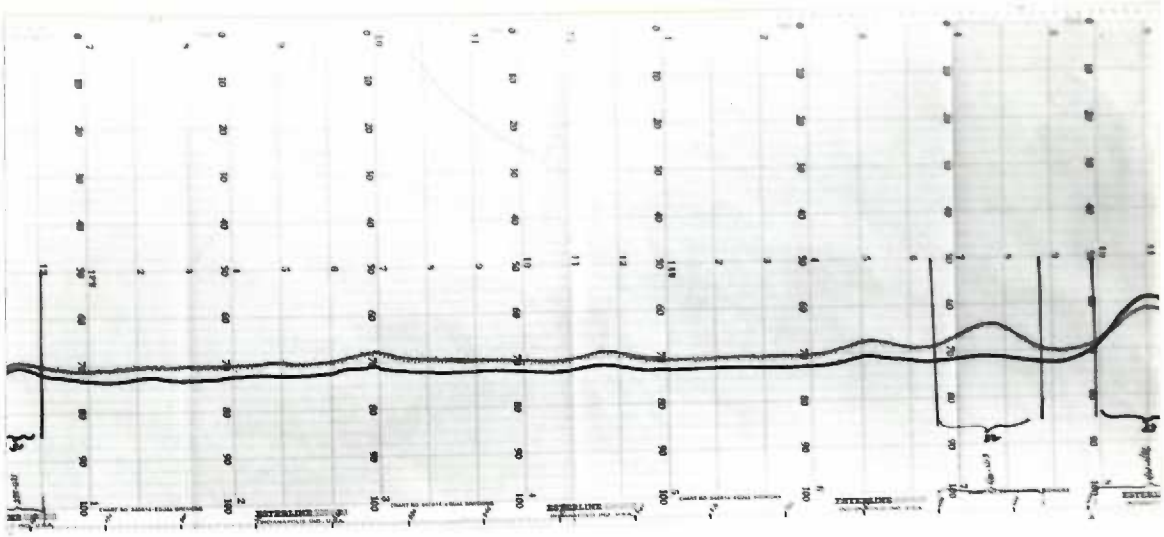

21

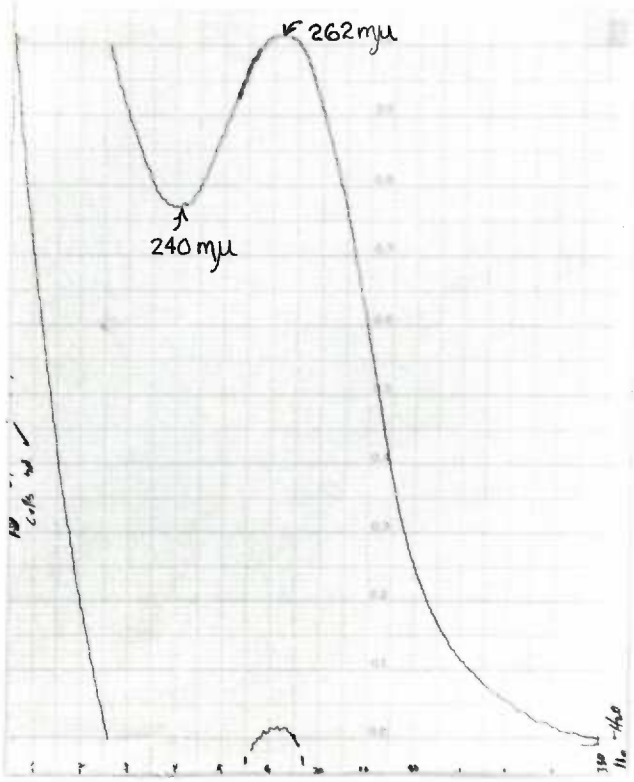
(Figure 24). A continuation of the profiles seen in Figures 20 and 22.

(Figure 25). A continuation of the profiles seen in Figures 21 and 23.

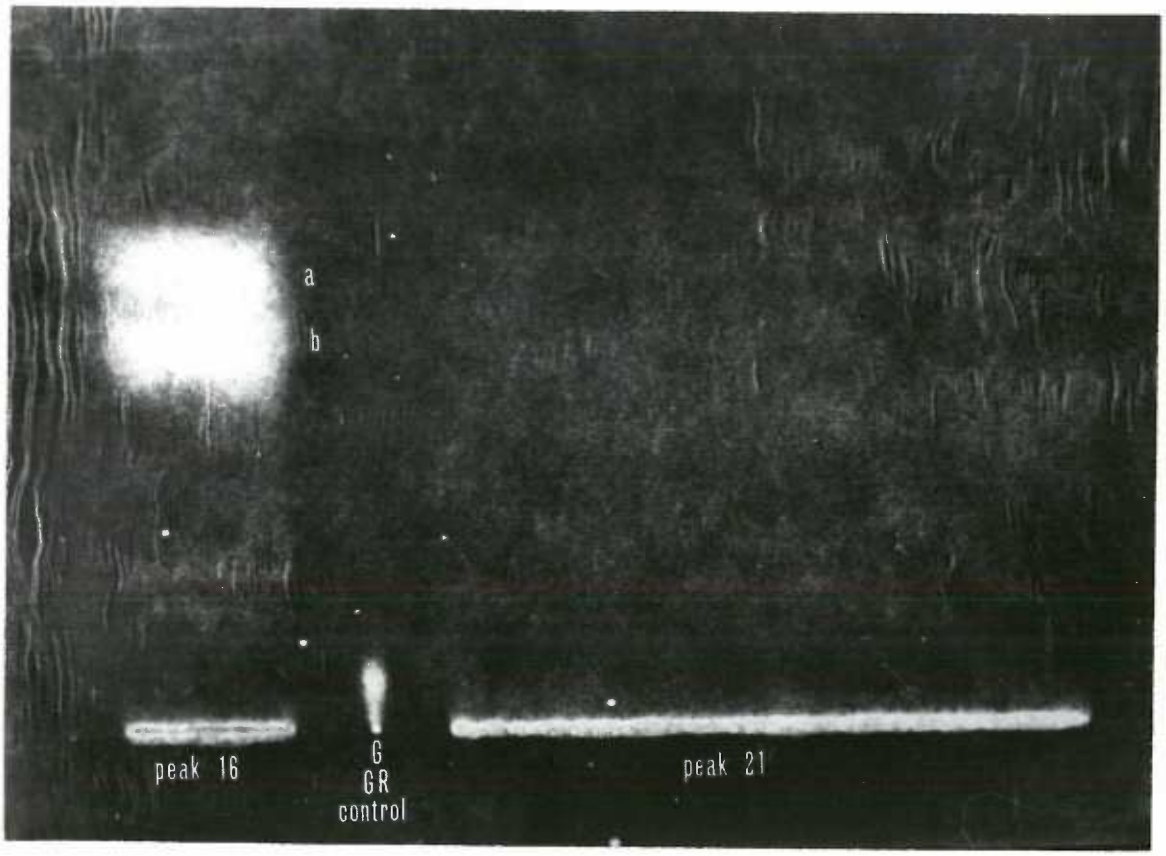




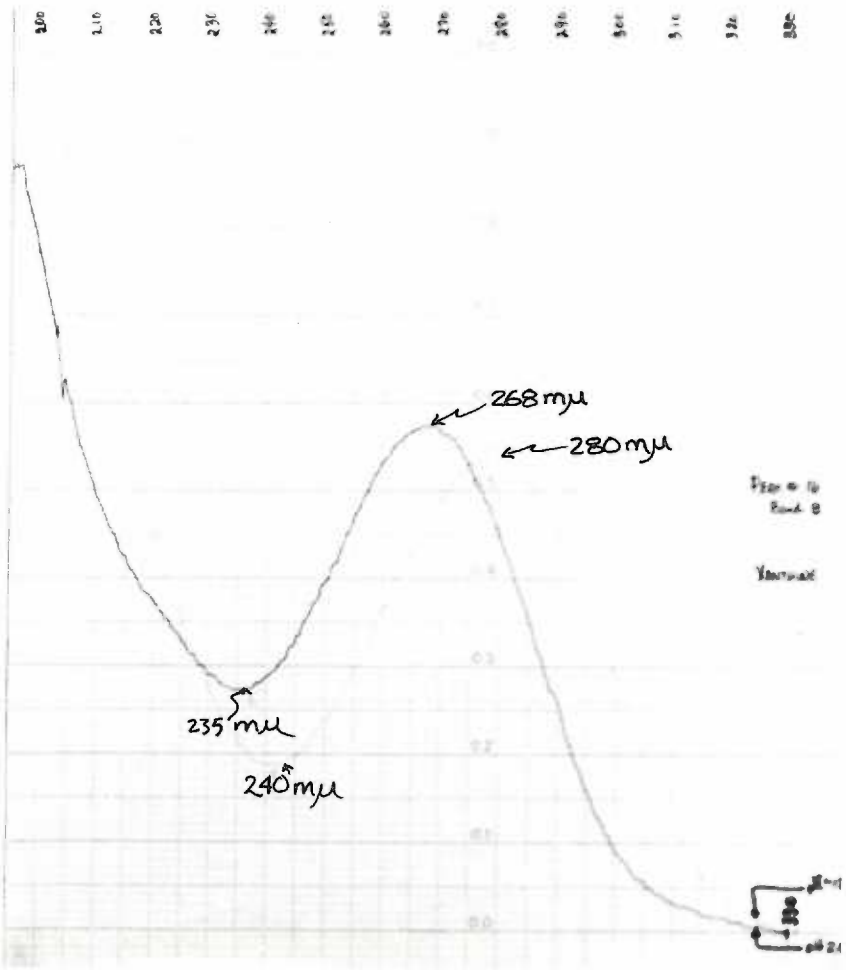
(Figure 26). Shown is a spectrum of peak 16 in glass distilled water run on a Carey, Model 15, spectrophotometer indicating a maximum absorbance at 262 mμ and a minimum at 240 mμ.


262mμ
240mμ

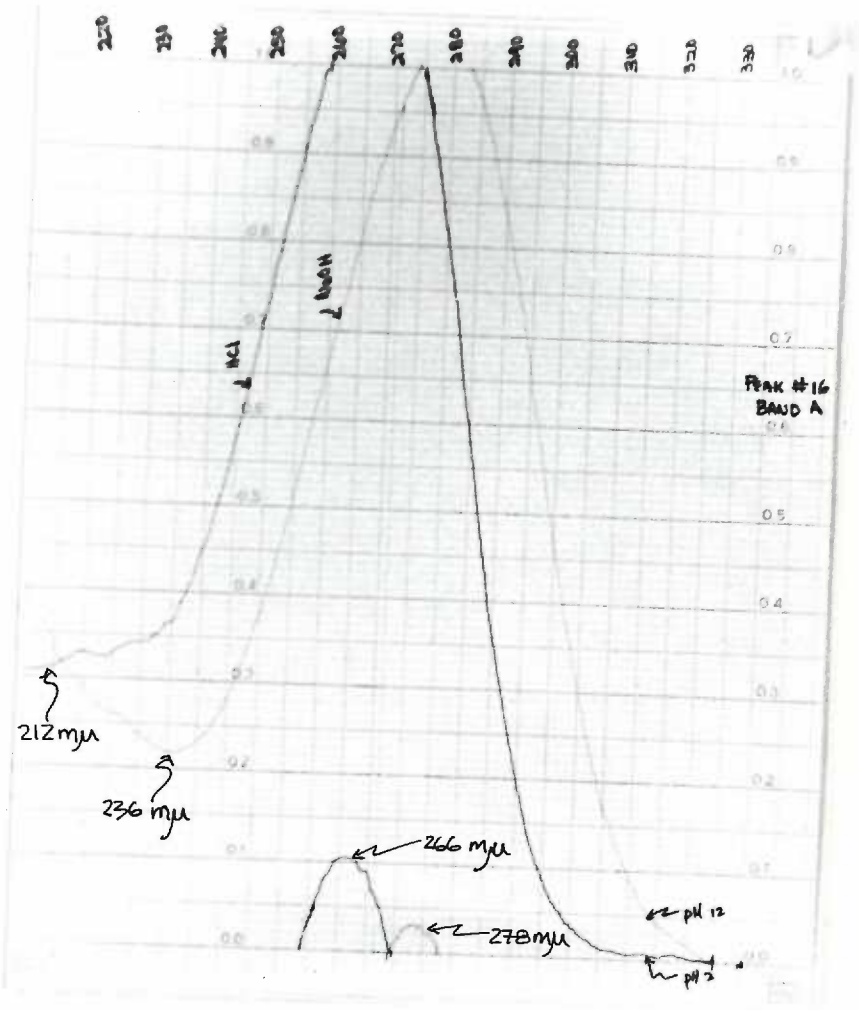
(Figure 27). A photograph of the paper chromatograph showing the separation of peak 16 into two components, "a" and "b", when placed in a butanol/water solvent.


a
b
peak 16
G
GR
control
peak 21

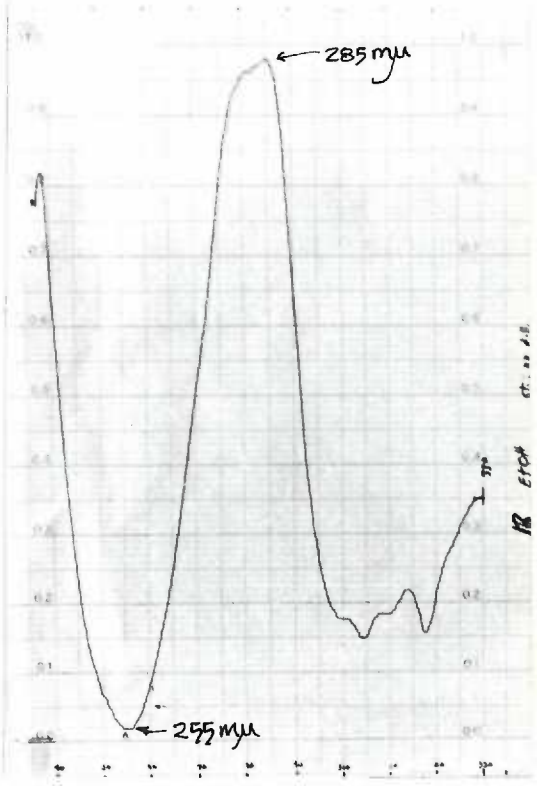
(Figure 28). A spectrogram of peak 16, component "b", showing the spectral shift under acid and alkaline pH. This component was identified as xanthine.


268mμ
280mμ
235mμ
240mμ

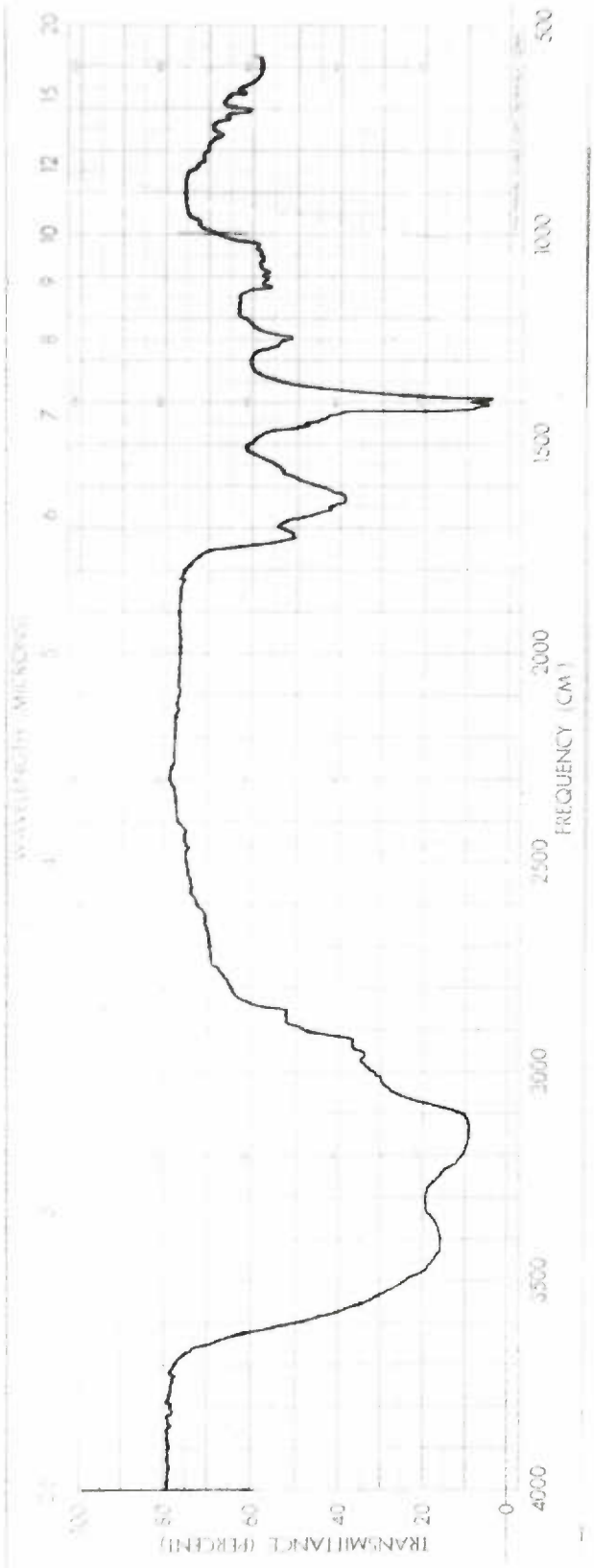
(Figure 29). A spectrogram of peak 16, component "a", showing the spectral shift under acid and alkaline pH. This component has not been identified.


PEAK #16
BAND A
212mμ
236 mμ
266 mμ
278mμ
pH 12
pH 2

(Figure 30). The spectrum of peak 13 was determined in 95% ethanol. Because of its maximal absorbance at 285 mμ and lack of solubility in water it was assumed not to be of an nucleic acid nature


285 mμ
255 mμ

(Figure 31). An infra-red spectrogram of the isolated peak 13 component was determined using the KBr pellet technique.


WAVELENGTH MICRONS
FREQUENCY (CM⁻¹)
TRANSMITTANCE (PERCENT)

DISCUSSION

A host-virus system was described by Rauscher in 1962 (6). This newly isolated and highly infective murine leukemogenic virus induced a biphasic type of disease. It was characterized by rapid and marked proliferation of primarily erythrocytic and leukocytic elements. In those surviving mice, the erythrocytopoiesis was followed by a lymphocytic leukemia beginning at 30-45 days post inoculation. Siegel and his associates (84), while investigating the pathogenesis of the virus induced disease, was able to demonstrate reticulocytosis, anemia, and increased nucleated cell count. They described the disease to be primarily an erythroleukemic response in mice to this virus which was reminiscent of the DiGuglielmo's disease in man (85). Many investigators have studied the effect of host-virus relationship upon nucleic acid metabolism and subsequent alterations in protein synthesis. To study this relationship, various models, such as, phage, tissue culture, and whole animal systems, have been used. The Rauscher model should present an interesting system for investigation of the pathogenesis of leukemia because of the reported dual nature of the progression of the disease (6).

The composition of urine in pathologic states generally differs from the normal in relative amounts of constituents. One chief object in studying the composition of urine is to obtain evidence, through the presence of abnormal substances or substances in abnormal amounts, that may be of aid in the diagnosis and treatment of pathologic states. Consequently, a study was considered in which changes in the nucleic acid pool, as reflected by urine, would be correlated with the pathogenesis of virus-induced murine leukemia.

Leukemic development was followed hematologically by cell counts and differentials. The data obtained from this present study confirms the earlier work of Siegel et al (84). The elution profiles of urine from infected mice was shown to evince marked differences with the temporal appearance and increase of peak 13 and elevation of peak 21. The other peaks in the elution profiles of infected mice did not appear to be sufficiently different from the controls. By virture of these observations, it was decided that pursuit, in greater detail, of the nature of peaks 13 and 21 may offer more significant information. It was this decision, based upon the observation of peaks 13 and 21, which altered the course of investigation from the original proposed study.

It was only very recently, upon attempting to elucidate the nature of the isolated fraction, that it was discovered that the isolated fraction was not a component of nucleic acid. Since the experiments were not designed for analysis of this type, only small quantities of material were available for characterization. Experiments must be designed and performed for the expressed purpose of obtaining substantial material from these peaks to do adequate characterization studies.

In this study, it was shown that these peaks did have a temporal correlation with the increase in total nucleated counts. Attempts to correlate the appearance and elevation of these peaks with lymphocyte, erythroblast, and polymorphonuclear leukocyte activity were obscured because of the existance of different pathological states of the mice from which pooled urine samples were obtained. A more definite correlation could be acquired if elution profiles of urine obtained from individual animals could be serially followed throughout the

course of the disease.

Weaver (99) has observed an inverse relationship existing between hemoglobin and total nucleated cell counts in mice infected with the Rauscher leukemogenic virus. This would imply that as the nucleated cell counts increased that there would be a concomitant decrease in the hemoglobin. If the anemia is due to increased loss and destruction of red cells, one may speculate that this may be a possible source of the component found in peak 13. Another possible source for the component may be due to cellular necrosis in the spleen because of its enlargment. The former speculation appears to be more plausible by virtue of the peak's close temporal correlation with the total nucleated cell count.

While complete data was not available to completely elucidate the nature and source of the components in peak 13 and 16, it is not beyond reason to consider investigation of these peaks as a possible parameter to provide further insight into the problem of pathogenic development of virus-induced leukemias.

SUMMARY AND CONCLUSIONS

Chromatographic elution profiles of urine from Balb/c mice infected with Rauscher leukemogenic virus were serially followed for 16 weeks. Data was obtained from three groups of animals, Group A, B, and C, consisting of 8 mice each. Groups A and B were inoculated with the leukemogenic virus while Group C served as control. Blood samples from each group were drawn weekly via the ophthalmic venous plexus and concurrent nucleated blood cell counts and differentials performed. Pooled urine samples were collected from each of the groups by specially constructed metabolism cages to avoid fecal contamination. Chromatographic separation of urine was achieved through ion exchange column chromatography using Anderson's continuous spectrophotometric column monitoring system. Other methods, such as, ultra-violet spectroscopy, infra-red spectroscopy, and paper chromatography were used to partially characterize the pertinent isolated fractions.

The results of these studies with a virus-induced murine leukemia demonstrate chromatographic elution profiles of urine to be an indicative parameter of the state of pathogenesis. A correlation of the relative concentrations of two unknown peaks with the total number of nucleated cells has been shown to exist in the infected animals. The exact relationship to the two peaks to the pathogenesis of viral infection and the nature and source of these two components are presently unclear and need further investigation.

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APPENDIX

APPENDIX A

Metabolism cage

(a) Design and construction of collection unit. A unique feature of this metabolism cage lies in its simplicity of design and construction. The design was such that the configuration and size may be readily altered to conform to the needs of the investigator without loss of separation efficiency. Additional advantages are that no special tools are required for construction and the unit was relatively inexpensive to build.

The collection unit is seen in Figure 1. The rationale behind this design was as follows. The urine drops to the accumulator plate and flows downward, drips onto the separator plate and flows downward. It then drips onto and around the collector tube and finally accumulates in the urine sample holder. The feces, on the other hand, falls down to the accumulator plate and ricochets off the separator plate, subsequently falls to the side and to the bottom of the collection unit. A stainless steel screen was placed over the urine sample holder to prevent any accidental fecal contamination.

Materials needed for the construction of the collection unit are: 1/4-in thick Plexiglas, Pyrex glass tubing, stainless steel screen (approximate mesh size, 16 squares per inch) and Plexiglas cement (ethylene dichloride or chloroform may be substituted). The amount of material required is governed by the size of the collection unit.

After cutting the Plexiglas to appropriate dimensions, the four sides of the unit were cemented together and allowed to set. The accumulator plate was then cemented into place. The accumulator plate should be placed into the unit at as an acute angle as possible to assure good urine flow with minimal fecal sticking. The separator

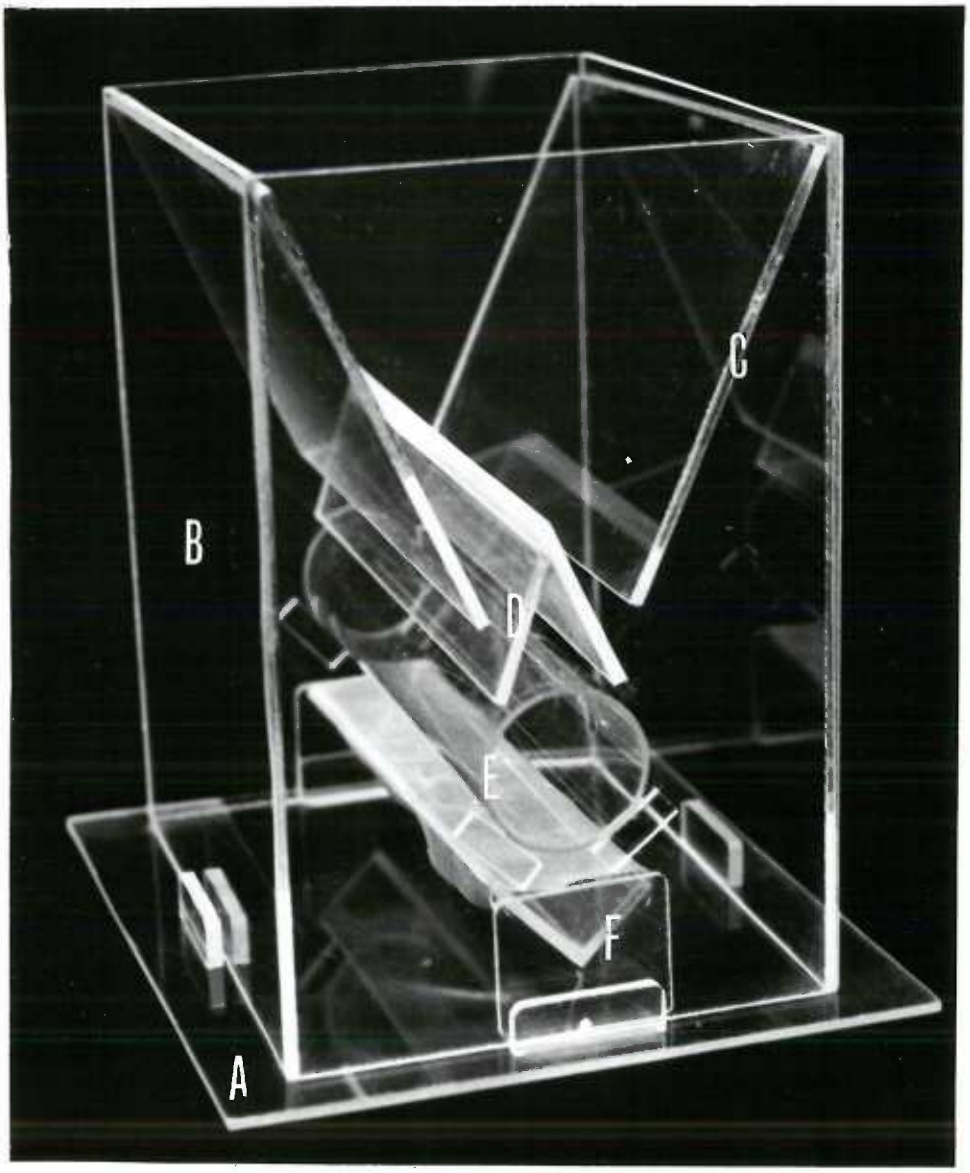
plate was cemented into place after making sure that the base, the widest portion, would lie slightly beyond the limits of the bottom end of the accumulator plate. This alignment is critical, because, if the base portion of the separator plate does not lie beyond the limits of the bottom end of the accumulator plate, the urine will drip directly to the bottom of the cage and will be lost. Finally, the collector tube (Pyrex) was anchored into place with four Plexiglas cleats which were cemented to the front and back side of the unit. The tubing selected should be of a diameter slightly larger than the width of the base of the separator plate.

The maximum distance from the bottom of the collector tube to the top of the urine sample holder should be approximately one inch.

The urine sample holder was essentially a trough constructed of Plexiglas with two Plexiglas end plates cemented into place (Fig. 2). The stainless steel screen was capped over the holder to prevent any feces from falling into the collected urine.

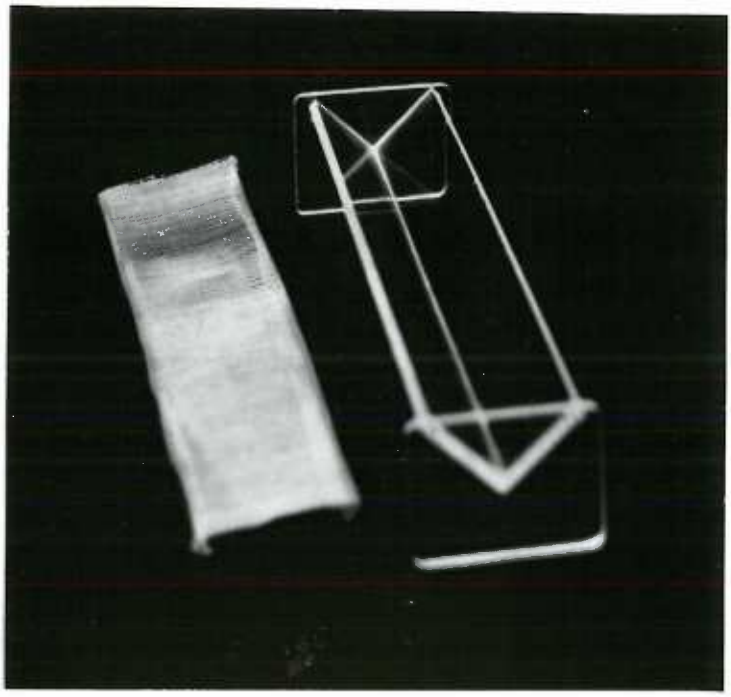
(b) Modification of the animal cage. A standard 7-in x 7 1/2-in x 10-in stainless steel cage with a screen bottom was modified by the addition of a water trap to prevent water from the watering bottle to flow into the collection unit and by the addition of a feed hopper which was located out away from the cage (Fig. 3). The feed hopper was constructed of Plexiglas so that only one animal at a time could feed, this was necessary to prevent mice from staying in the hopper chamber. The bottom of the feeder unit was made of 1/4-in mesh screen which allowed food particles to drop outside of the collection unit by way of avoiding contamination of the collected urine sample.

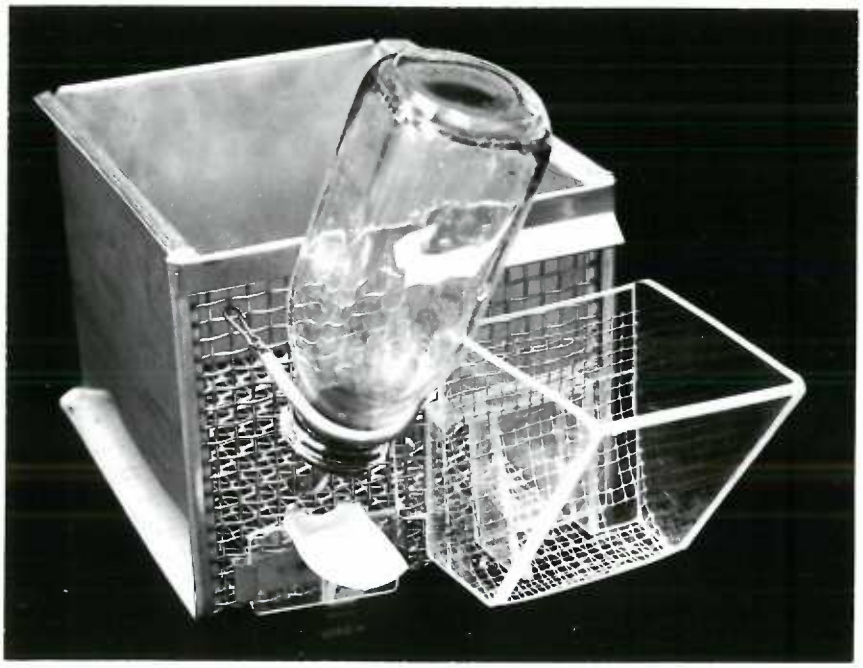
(Figure 1). Pictured is the Plexiglas urine collection unit designed and constructed in this laboratory. The major features which can be seen are the base plate (A), and the colection unit (B) which consists of the accumulator plate (C), separator plate (D), collector tube (E), and urine sample holder with stainless steel cap (F).


A
B
C
D
E
F

(Figure 2). Shown is a closer and more detailed view of the construction of the Plexiglas urine sample holder and stainless steel screen cover.

(Figure 3). A modified animal cage with the specially constructed feed hopper and water trap is placed upon the urine collection unit during the urine collection.





APPENDIX B

Modification of centrifuge

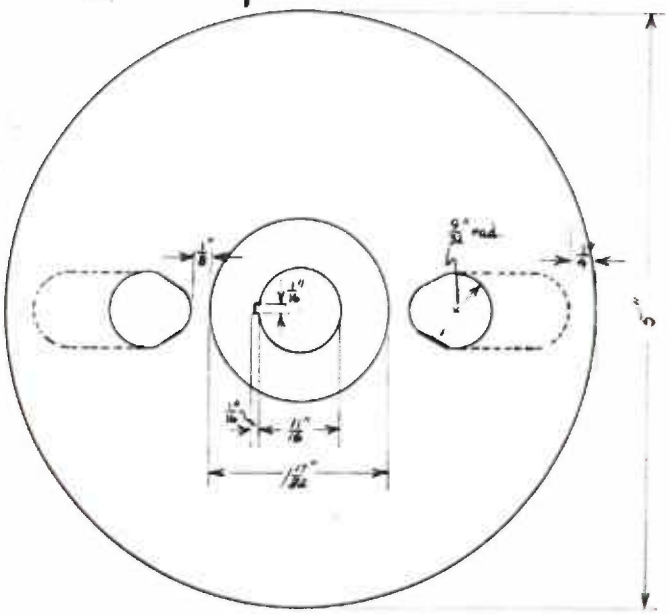
A small two cup head was designed and constructed in this laboratory for the Wifug, Rastgeldi type, centrifuge. This modification permitted the holding of the 3 dram screw cap vials in which the urine samples were routinely stored (Fig. 4).

A cover, normally used on continuous centrifugation heads, was thoroughly greased with silicone grease (Dow-Corning) and the hole in the center of the cover sealed with a rubber stopper. This cover was used for a mold for the fabrication of the newly designed centrifuge head. After the mold had been greased, Epon 812 (Shell Oil Company) plastic was poured into the mold to a depth of approximately 2 inches, then placed into a vacuum oven (National Appliance Company) and subjected to 25-30-in Hg vacuum at 60°C. The vacuum was necessary to remove all bubbles from the Epon plastic. The head was "cured" for a week at 60°C to minimize and eliminate all stress lines. After the head had been "cured", the head was then "trued" and statically balanced on a machine lathe; the two vial holes were drilled at a 45° angle while clamped to a milling tool. Following the various construction steps, the Epon centrifuge head was returned to the vacuum oven at 60°C and permitted to anneal for four days, to eliminate any additional stress lines acquired in the lathing and milling processes.

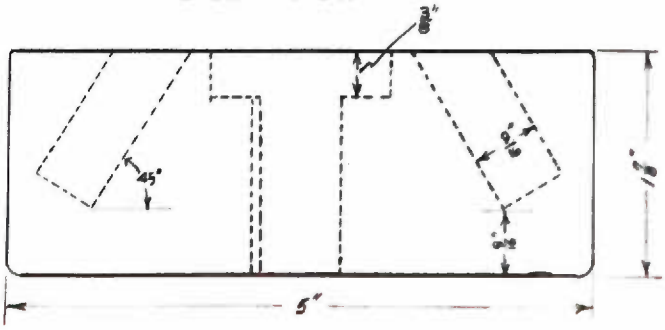
The completed head (Fig. 5) was placed in the aluminum cover, the cover originally used for the mold, and positioned onto the centrifuge (Fig. 6) for use. This cover offered additional protection to the user while in operation.

(Figure 4). Shown is the dimensioned drawing of the Epon 812 centrifuge head designed and constructed in this laboratory.

Top View



Side View



(Figure 5). A photograph of the finished Epon 812 centrifuge head which holds two 3 dram screw cap vials in which urine samples were routinely stored

(Figure 6). The Wifug, Rastgeldi type, centrifuge with the Epon 812 centrifuge head enclosed in the aluminum protective cover is used to centrifuge the prepared urine samples.

APPENDIX C

Continuous double-beam spectrophotometric column monitoring system

A detailed description of the nucleotide analyzer developed by Anderson (96) and as set up in this laboratory is presented in the following text. A photograph of the complete system was shown in Figure 1 of Materials and Methods. The system used in this investigation was very similar to Anderson's system except for a few minor alterations in the instrumentation. A schematic flow diagram of the analyzing system is presented in Figure 7.

Three 600 ml Pyrex gradient cylinders were connected in series. A magnetic stirring bar was placed in the first cylinder and stirred continuously during the analysis by a magnetic stirrer (Magnestir, Cat. No. 58945-1, Van Waters and Rogers, Inc.). A 250 watt infra-red lamp was used to slightly heat the first cylinder in order to reduce the level of dissolved gases. The first gradient cylinder was connected to a reference flow cell (Beckman Instruments, Inc., Model 97290) (Fig. 8) by Teflon tubing.

The modified Beckman DB spectrophotometer used in Anderson's system was further modified in this investigation (Appendix D). A small D.C. motor, geared to the wavelength dial, moved the dial alternately from 260 to 280 mμ in synchrony with the printing cycle of the Esterline-Angus, Model 1124-E, multipoint recording potentiometer.

The hydraulic pump used in the system was the Milton-Roy chromatographic pump (Model 196-31 Simplex instrument minipump, Milton-Roy Company). Connections to the pump were made with 1/8-in Teflon tubing through Spinco chromatography stainless steel fittings. All male threaded parts of connectors and fittings were wrapped with Teflon

tape prior to threading to avoid leakage at these connections.

The pressure gage (Marsh Instrument Company), graduated in units of 10 from 0-600 lbs/in^2, was used in the Teflon line between the pump and column to indicate column pressure. The gage was isolated from the buffer system through a stainless steel gage isolator (Mansfield and Green, Type B-5, Cleveland, Ohio) to prevent not only damage to the pressure gage but also to prevent contamination of the buffer.

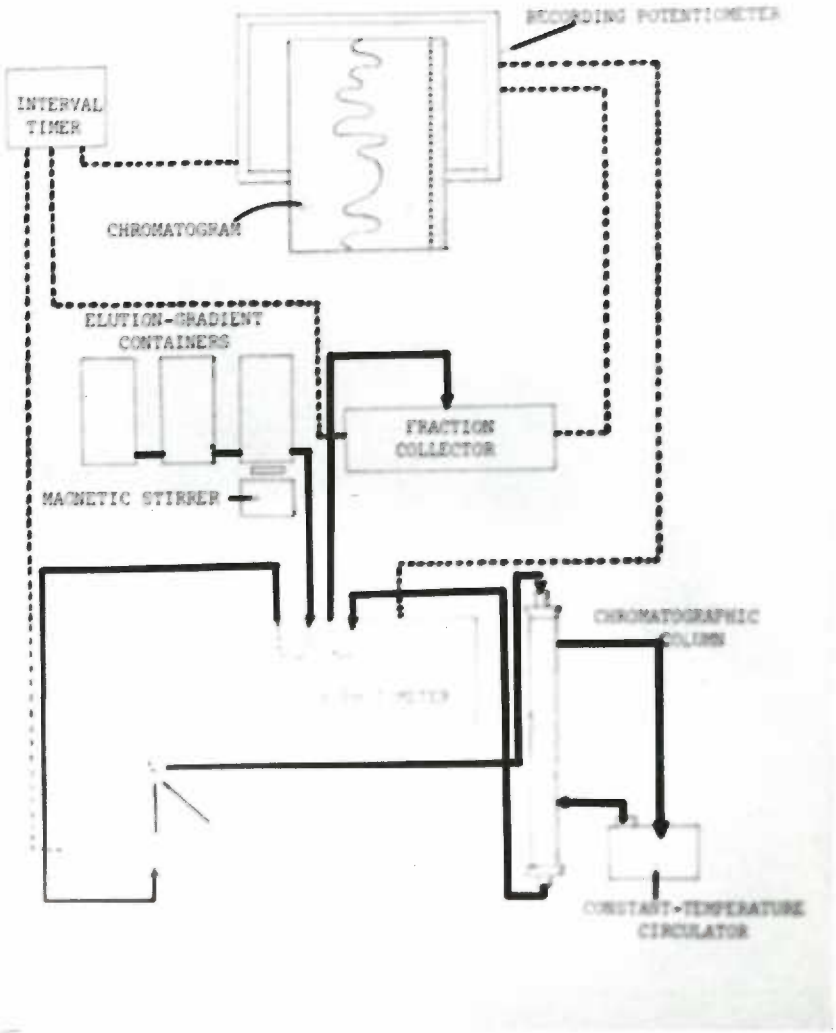
Connections from the pump to the top of the chromatography column was through a polyvinyl chloride plunger fitten with Buta-N rubber "O" ring seals. These seals must be replaced periodically as they become brittle and lose their sealing capacity when subject to exposure to the buffer system. The seals were lightly greased with Dow-Corning silicone grease prior to being put into place in the column. Because of the hydraulic pressure exerted on the column, the plunger must be clamped on to prevent leakage. The column was jacketed and had a glass frit column of 0.9 cm i.d. Column temperature was controlled with a circulating constant temperature bath. All fluid lines beyond the column were of 1/16-in o.d. Teflon tubing.

The fractions were collected by a TMC Vanguard, Model 1010, time-drop fraction collector mounted in a refrigerated cold air box at approximately 1-2°C. A digital counter was connected to the fraction collector to indicate the tube fraction number. The counter was also connected in series with the event marker of the recorder to indicate each cycle of the fraction collector.

The power cables from the recording potentiometer, fraction collector, and hydraulic pump were connected to an interval timer (Paragon Time Control, Model 1015-0S) to automatically start or stop

the chromatographic fractionation.

(Figure 7). A schematic flow diagram of the continuous column monitoring system used for analyzing ultra-violet absorbing constituents of urine.


RECORDING POTENTIOMETER
INTERVAL TIMER
CHROMATOGRAM
ELUTION-GRADIENT CONTAINERS
FRACTION COLLECTOR
MAGNETIC STIRRER
CHROMATOGRAPHIC COLUMN
CONSTANT-TEMPERATURE CIRCULATOR

(Figure 8). A photograph of the Beckman, model 97290, flow cell used as the reference and sample cells in conjunction with the modified Beckman DB spectrophotometer.

APPENDIX D

Multiple-wavelength selector modification (100, 101)

A versatile, automated analytical system for quantitating proteins, peptides, and nucleic acid derivatives had been described by Anderson (96). In that system, absorbance at one or two wavelengths was continuously monitored and recorded. The two wavelengths in Anderson's dual-beam monitoring system were positioned by an air piston driven mechanical device which may offer some disadvantages. For example, wavelength selection was limited to the stroke length of the air piston, and air lines with pressure gages and regulators were required. Also, the time spent in assembly and disassembly of the modification to the spectrophotometer could be lengthy. An inexpensive electronic wavelength selector is described which, while performing the same function, eliminates these drawbacks in the Anderson system.

Instrumentation

A two-point scan system was a modification of the pneumatic system described by Anderson (96). A spectrophotometer with a recording output provision was made to scan between two selected wavelengths which were time-locked to a multipoint recorder. This time sharing of the recorder permitted the operator to superimpose the simultaneous records of the absorbance of the two wavelengths.

Figure 9 represents the schematic for the system. Two filament transformers, with their secondaries connected in series, provided AC voltage which was rectified to produce both a positive and a negative DC voltage to power the motor. The positive voltage caused the motor to rotate in one direction while the negative produced the opposite rotation. The voltages were connected to the motor through the auxiliary switch deck [1] of Section A in the recorder.

Section A of the added switch was wired so that the motor which drives the wavelength scanner was supplied alternately with a positive and a negative voltage. When the motor reached the desired limit, one of the selected wavelengths, the micro switch was opened interrupting the current. When the rotary switch moved to the next position, the opposite polarity voltage caused the motor to move to the opposite wavelength limit switch.

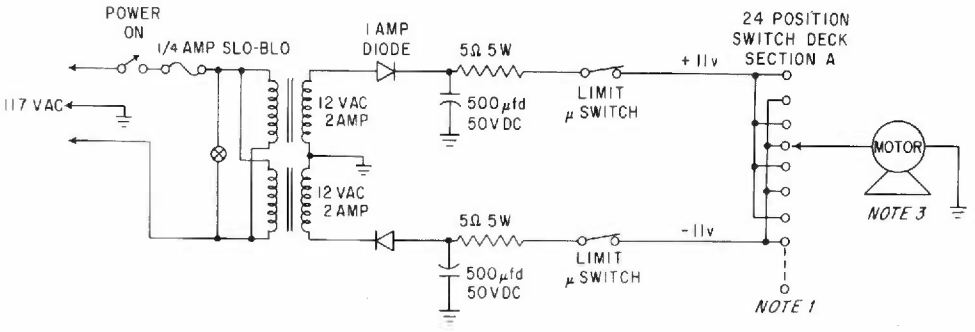
The modification of the spectrophotometer[2] necessitated the removal of the old motor and motor plate. The new motor plate assembly (Fig. 10) included the two limit micro switches. Two limiter clamps were attached by set screws for the desired wavelengths. If connectors were placed on the wires to the old motor plate assembly, the system could be returned to its original configuration in less than 15 min when unidirectional scan was necessary.

The signal from the spectrophotometer output was connected to the master sensitivity (Fig. 9 and 11). The signal from this adjustment was connected to both the reversing switch and the balance adjustment control. These adjustments allowed the operator to set the incoming level and to equalize the output levels. The output from the balance adjust was also connected to the signal reversing switch. The signals were connected to the recorder through Section B of the rotary switch. The recorder was alternately supplied with signals which were produced by the two selected wavelengths. Their amplitudes were equalized by the balance adjustment using a pertinent reference solution. This was

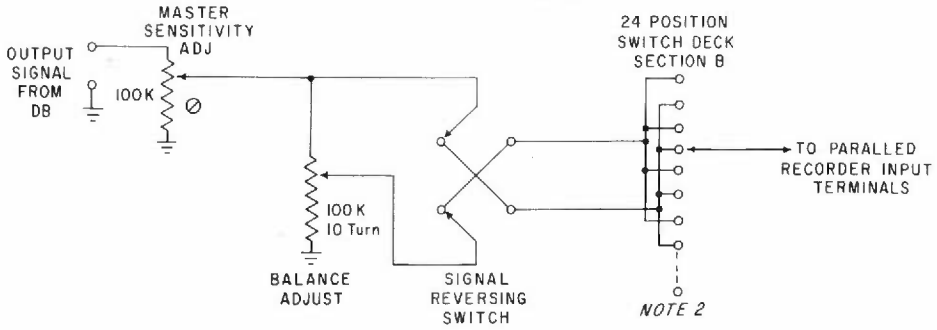
[1]The auxiliary switch deck was a factory modification by the manufacturer of the recorder. It had two poles and twenty-four positions for each pole. Its rotation was coincident with the recorder scanning of each of its twenty-four input points.

[2]Beckman model DB spectrophotometer.

(Figure 9). A schematic representation of the electronic modification involved in the electronic wavelength selector mechanism.

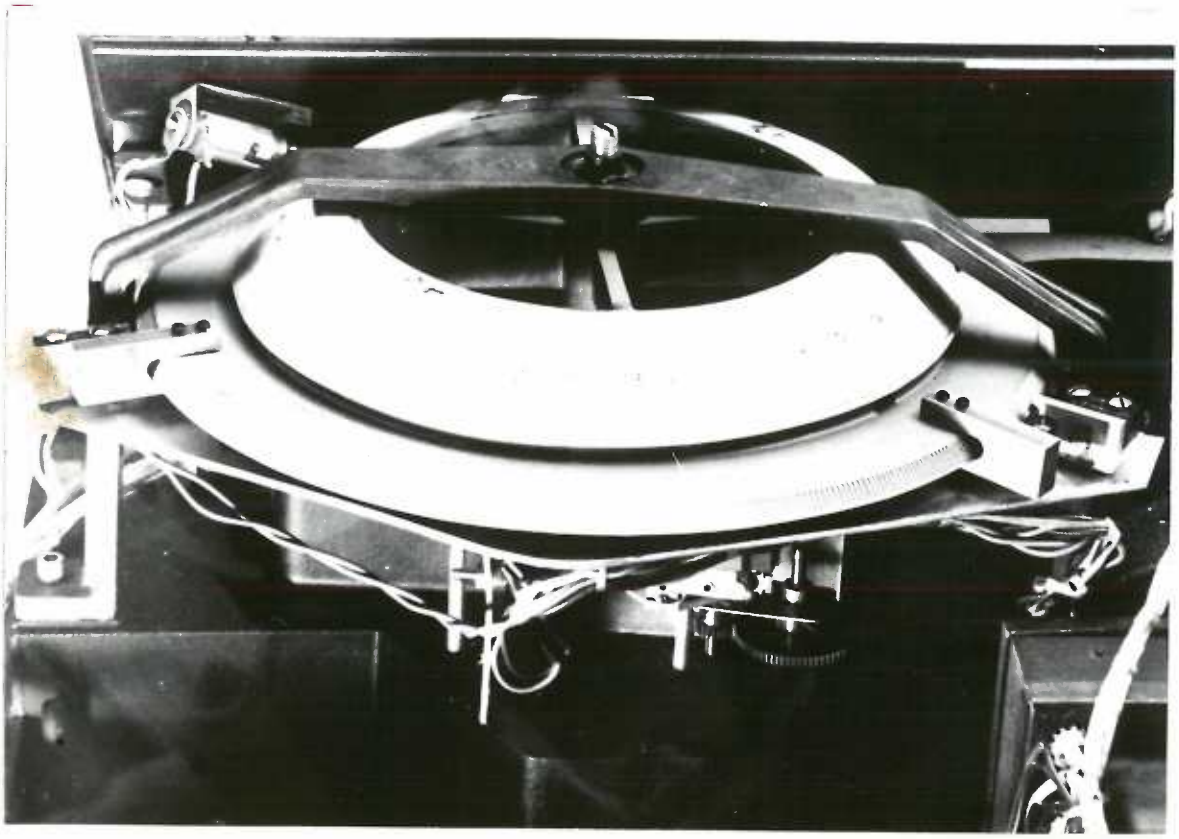


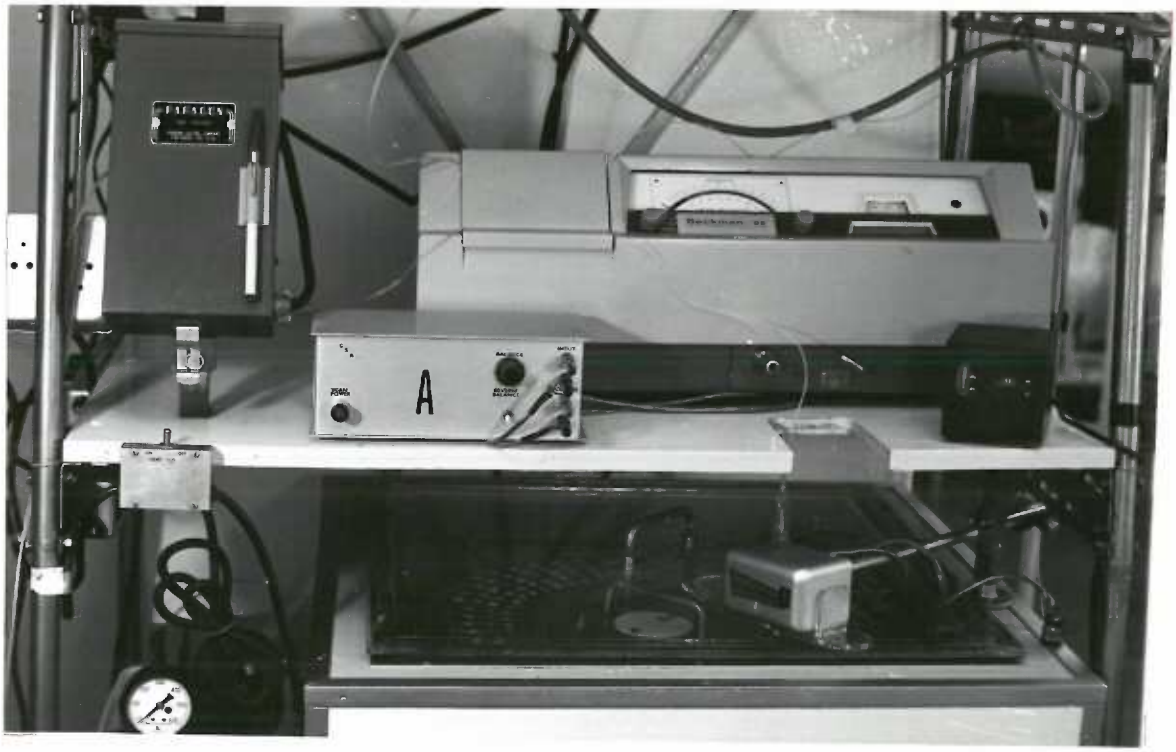
NOTE: 1. Factory modification: Aux. switch deck added to input selector switch frame, Section A. Esterline Angus Recorder E1124E
2. Same deck as Note 1, Section B.
3. Pittman DC motor, model DC706, reduction gear train added.



(Figure 10). Photograph of the modified motor plate with limit micro switches.

(Figure 11). The master sensitivity control unit (A) is used to adjust the incoming signal level and equalize the output level.




A

accomplished by adjusting the spectrophotometer output and the master sensitivity adjustment. Through the use of these two controls, one sets the lowest reading to equal 100 per cent on the recorder. The greater signal was then adjusted to read 100 per cent on the recorder by using the balance adjustment control. The reversing switch allowed the operator to synchronize the selected wavelengths to the point recorder.

This system had a further advantage of lower cost as opposed to the pneumatic system. Also, greater scan widths could be employed, up to 180°, limited by the internal construction of the spectrophotometer and was readily readjustable for different wavelengths.