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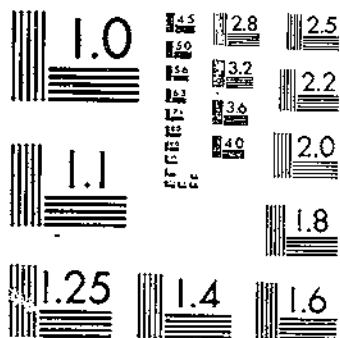
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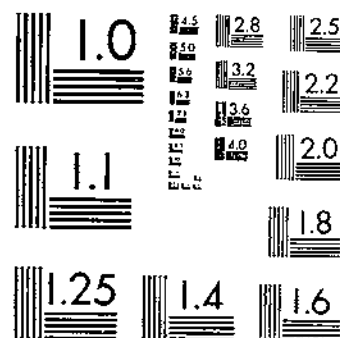
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THE DIFFERENTIAL DIAGNOSIS OF LYMPHOID LEUKOSIS AND MAREK'S DISEASE
SICCARDI, F. J. BURMESTER, B. R. U. 1 OF 1

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The Differential Diagnosis of Lymphoid Leukosis and Marek's Disease

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CONTENTS

	Page
Lymphoid leukemia	2
Marek's disease	3
Diagnostic guidelines	11
Age of occurrence	11
Neural signs and lesions	12
Involvement of bursa of Fabricius	12
Cytological differences in LL and MD tumors	18
Discussion	20
Summary	21
Literature cited	22
Appendix—Procedures for preparing and staining tumor smears	23

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The Differential Diagnosis of Lymphoid Leukosis and Marek's Disease

By FRANK J. SICCARDI and B. R. BURMESTER, *Regional Poultry Research
Laboratory, Agricultural Research Service*¹

Avian leukosis signifies a group of diseases in chickens characterized by an uncontrolled, excessive proliferation of immature erythroid, myeloid, or lymphoid cells. It includes erythroblastosis, when the red blood cells are affected, and myeloblastosis, when the myeloid or the granule-containing cells are affected. Often there is a combination of these two disease conditions.

The third and most common type of avian leukosis involves the lymphoid cells. Formerly this disease was referred to as lymphomatosis—neural, visceral, or ocular depending on the location of the principal lesion. Extensive studies (2, 8, 14)² recently have shown that excessive proliferation of the lymphoid cells is the primary lesion in two distinct diseases, both of which commonly occur and have been included under lymphomatosis. It thus becomes necessary to designate each disease.

A new classification and nomenclature were adopted at the Conference of the World's Veterinary Poultry Association at Bristol, England (1). In the United States they were recommended for adoption at the Technical Workshop Conference on Diseases of the Avian Leukosis Complex (3). The terms now used are lymphoid leukosis (LL) for the "big liver" disease, which is caused by an RNA myxo-type virus, and Marek's disease (MD) for the syndrome caused by a DNA herpes-type virus, which results in lymphoid accumulations in the nervous system, various visceral organs, eye, skin, and muscle.

Acute leukosis has been applied to disease outbreaks in young birds, in which there are lymphoid deposits in the nerves, viscera, and often the skin and muscle. This disease is also caused by the herpes virus of Marek's disease (15) and is now referred to by this term.

Since LL and MD tumors in the viscera of chickens are similar in gross and microscopic appearance, there is a problem of differential diagnosis. This bulletin presents information on pathogenesis and some diagnostic techniques for differentiating these two diseases. Methods based on detection of the causative viruses and antibody have been published (7, 18, 20).

¹ The senior author is now with the Campbell Institute for Agricultural Research, Fayetteville, Ark.

² Italic numbers in parentheses refer to Literature Cited, p. 22.

LYMPHOID LEUKOSIS

Lymphoid leukemia was formerly referred to as visceral lymphomatosis or big liver disease. It is a viral-induced lymphoblastic malignancy originating in the bursa of Fabricius (11).

Chickens become infected either congenitally via the egg or by direct contact with infected pen mates (4, 6). Resistance to infection is affected by a maternal antibody (5), but it is largely determined by at least three independent pairs of genes (12). Resistance to tumor formation is largely affected by other genetic factors (13).

Following infection of susceptible chickens, large amounts of virus are produced in many tissues without apparent harmful effects. Five to eight weeks after infection, lymphoid cells of one or more follicles of the bursa of Fabricius are transformed and the follicles become engorged with lymphoblasts (fig. 1). At this time these changes are microscopic, and grossly visible tumors of the bursa or of any other tissue are not observed.

The transformed bursa follicle remains relatively quiescent until or just before the chicken reaches sexual maturity. At about this time, i.e., 16 to 22 weeks old, the cells of the transformed bursa follicle, for reasons yet to be determined, start rapid division and acquire neoplastic characteristics (figs. 2 and 3). Con-

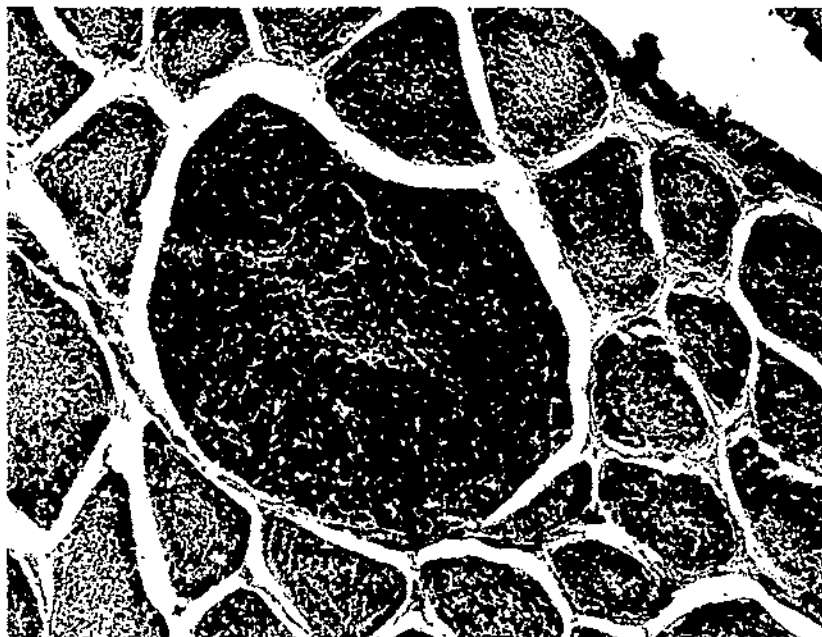


FIGURE 1.—Large follicle (center) has undergone neoplastic transformation because of LL virus. This is the first alteration. $\times 25$. BN-35246

currently the tumor cells spread from the bursa to other visceral organs, where focal or diffuse tumors may develop (figs. 4-7). Primary and metastatic tumors are both composed of a homogeneous population of neoplastic lymphoblasts (figs. 8-10; pl. 1, A).

Mortality from LL may occur as early as 16 weeks of age, but most losses occur when the chickens are older.

MAREK'S DISEASE

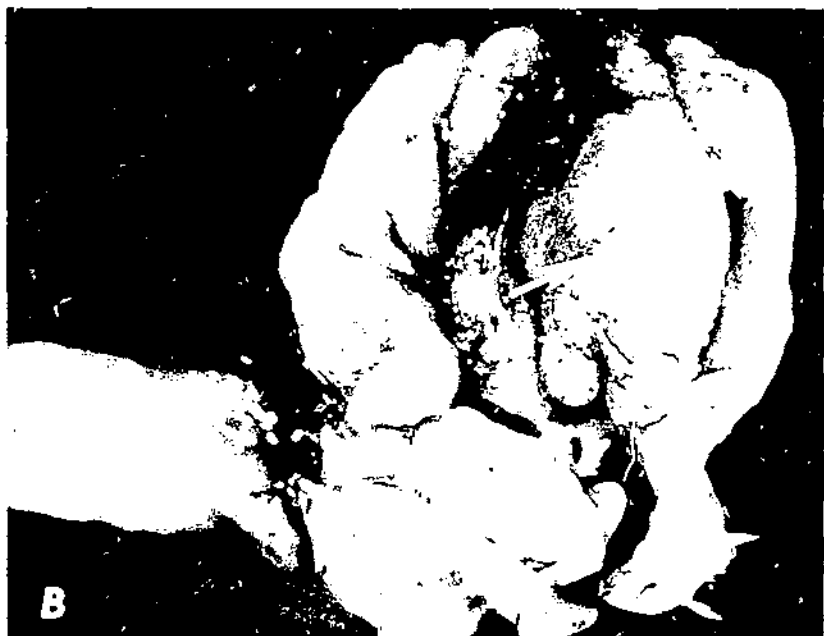
Marek's disease was formerly referred to as neural lymphomatosis, fowl paralysis, range paralysis, or acute leukosis. It is a viral-induced dyscrasia of the lymphoid cell system, resulting in lesions that range in type from what appears to be inflammatory to a malignant neoplasm. Recent research (8, 14, 19, 20) has provided strong evidence that a cell-associated herpes virus is the etiologic agent of MD.

The horizontal transmission of the infectious agent from infected chickens or contaminated environment to newly hatched chicks would appear to be the principal means of natural spread of the disease (10, 17). Although some evidence has been presented in support of egg transmission (16), it is far from conclusive. Factors other than the primary causative agent (herpes virus) may dramatically affect the extent of clinical manifestations of the disease.

Marked progress toward development of resistant and susceptible strains of chickens has been made by employing the usual principles of progeny testing and genetic selection (9). However, the number of genetic loci involved and their interrelationship have not yet been determined.

Lesions of MD may occur in a wide variety of tissues. The difference between lesions of MD and normal lymphoid foci (lymphoreactive area) is quantitative. The MD lesion, in comparison with the lymphoid foci, is larger and has a higher proportion of lymphoid cells with mitotic figures. Both contain small, medium, and large lymphocytes and few to many plasma cells. Another difference, especially in the rapidly growing visceral tumors, is that in the MD lesion the proportion of medium and large lymphocytes is often rather high; however, these proportions vary widely. This heterogeneity of cell type is characteristic and is in marked contrast to LL lesions, where the tumor cells are uniformly large, immature lymphoblasts (figs. 8 and 11).

In MD the location of the lesion also varies widely. Chickens of some flocks may show only neural involvement, whereas those of other flocks primarily have tumors of the viscera. Chickens of flocks in some areas vary markedly in the apparent predilection of the various visceral organs to the development of tumors. This variation has not been satisfactorily explained but may be due to the causative virus, genetic constitution of the chicken, unknown factors, or a complex interaction of two or more of these variables.





BN-35246, BN-35247, BN-35248

FIGURE 2.—LL tumors in bursa of Fabricius: *A*, Small; *B*, moderate size; *C*, hemorrhagic. Such lymphoid tumors in bursa of Fabricius are diagnostic for LL.

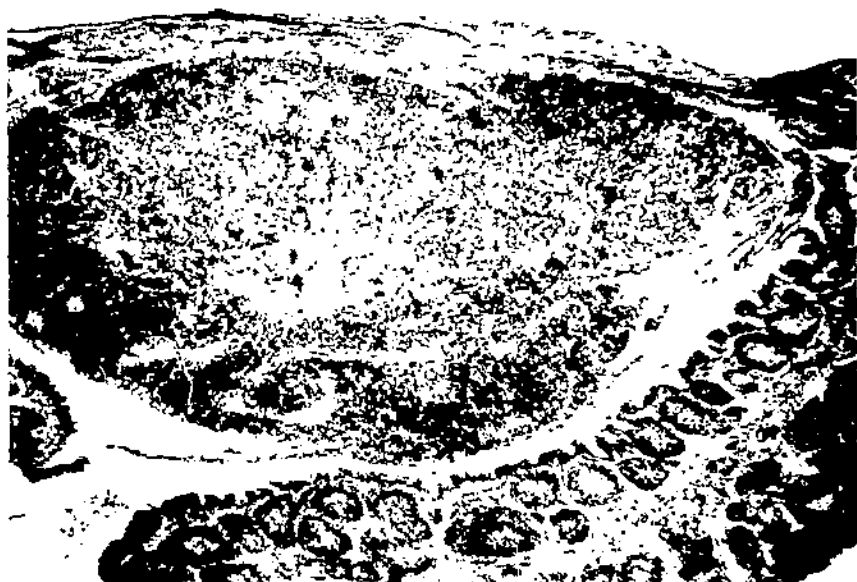


FIGURE 3. Focal L.L. tumor in bursa of Fabricius with adjacent normal regressing follicles. BN 35249
× 20.

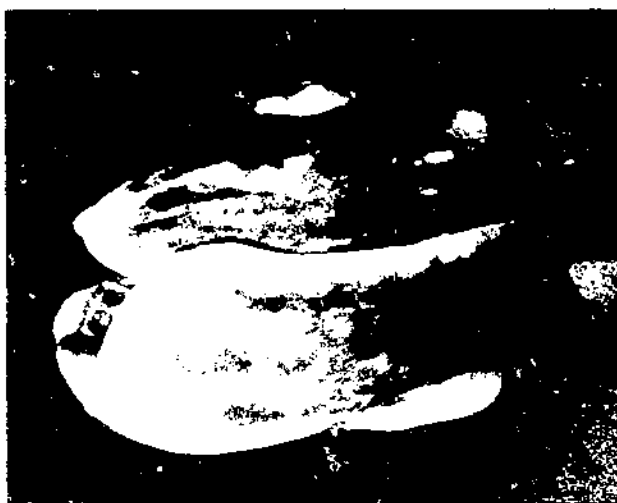


FIGURE 4. L.L. tumor of liver with grossly diffuse involvement. BN 35259

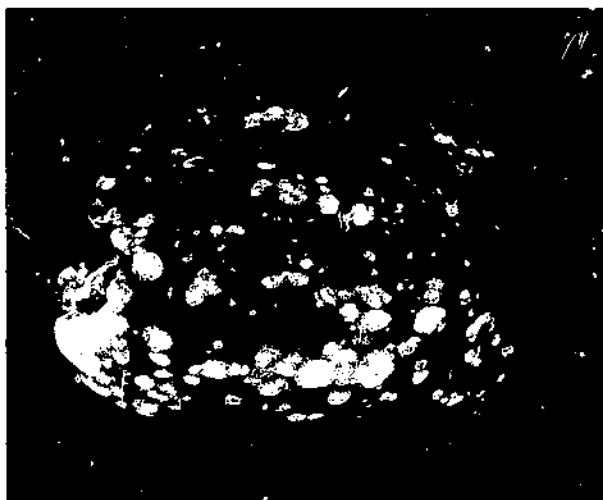


FIGURE 5.—LL of liver with focal or nodular tumor.

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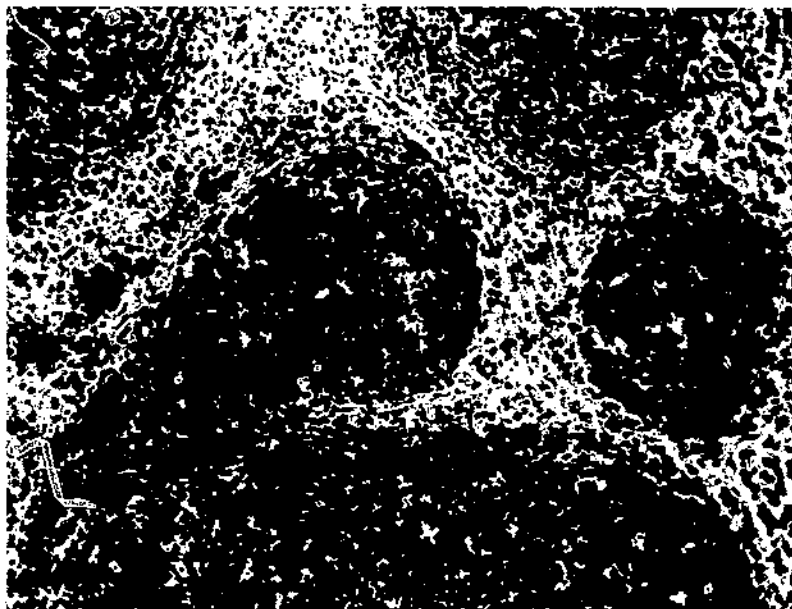
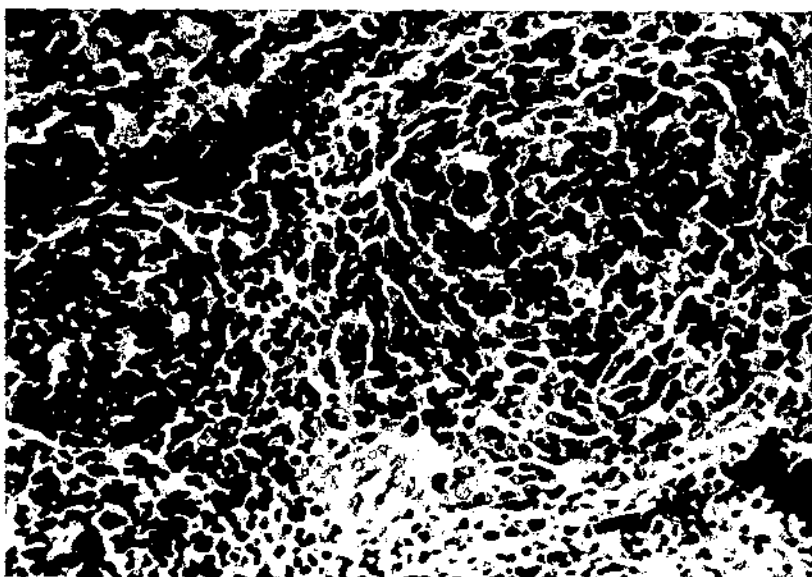
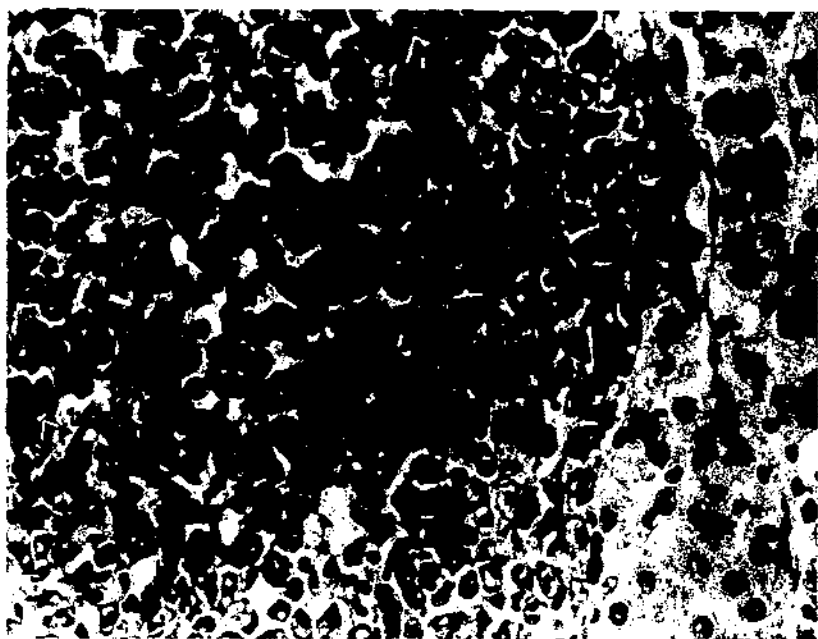


FIGURE 6.—Liver with LL. Lesions are multicentric. Centers may be so numerous as to give a gross appearance of miliary or diffuse involvement, or there may be only a few centers resulting in nodular tumor distribution. $\times 150$.

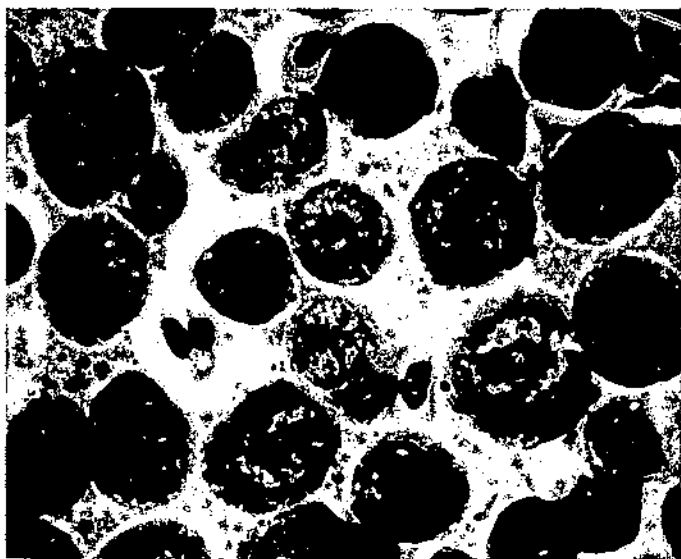
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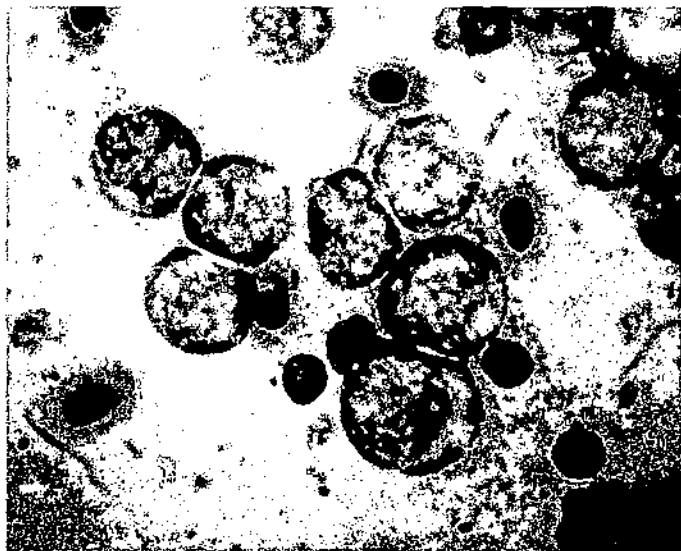
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FIGURE 7.—Spleen with LL lesions. Normal germinal center (left) is circumscribed with a fibrous capsule, whereas LL tumor (right) has no limiting membrane and is made up of lymphoblasts. $\times 400$.



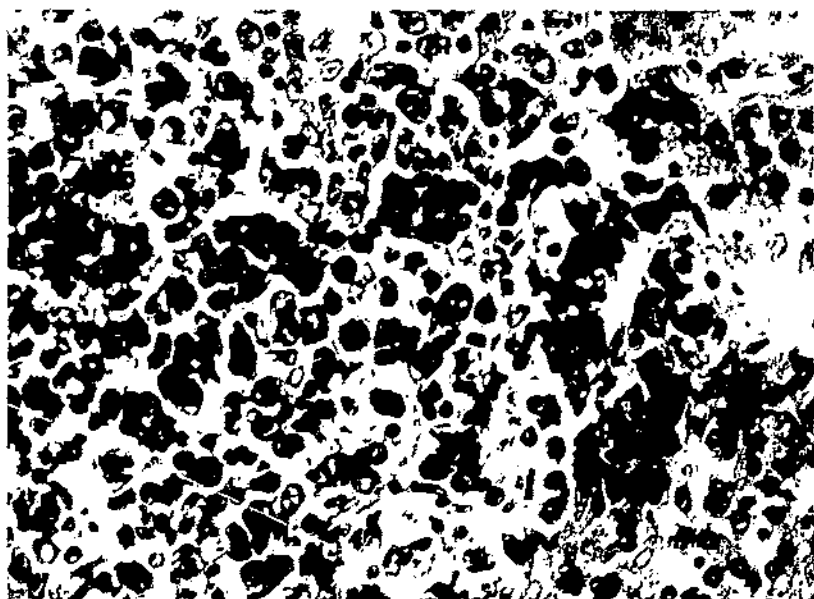
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FIGURE 8.—Liver with LL tumor. Lymphoblasts are uniform in size and morphology, with a vesicular nucleus and a prominent nucleolus. $\times 700$.



BN-35255
FIGURE 9.—Wet-fixed smear of LL liver tumor stained with hematoxylin. Note homogeneity of lymphoid population with mostly lymphoblast-like cells. Note also large cytoplasm-nuclear ratio, scanty nuclear chromatin, and prominent nucleolus, in contrast to small and medium lymphocytes in figure 12. $\times 2,000$.

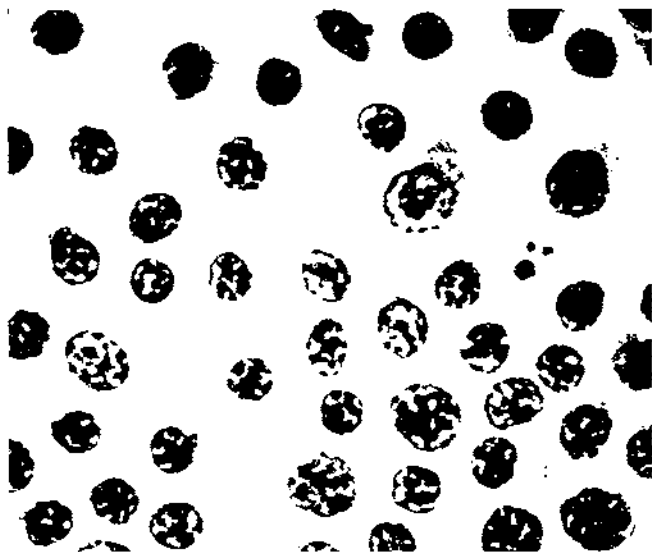


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FIGURE 10.—Dry-fixed smear of LL liver tumor stained with Wright's stain. $\times 2,000$. Note lack of nuclear detail, as contrasted to wet-fixed preparation in figure 9.



BN 35257

FIGURE 11. Tumorous liver of chicken with acute MD. Note variation in size and morphology of cells. Nucleoli of most cells are not detectable. This specimen has unusually large number of lymphoblasts, indicating high level of malignancy. $\times 700$.



BN 35258

FIGURE 12. Wet-fixed smear of MD ovary tumor stained with hematoxylin. Note heterogeneity of lymphocytic cells, with small to medium lymphocytes predominating. $\times 2,000$.

DIAGNOSTIC GUIDELINES

Many factors should be considered in the differential diagnosis of LL and MD, including (1) age of the chicken when disease occurs, (2) neural signs and lesions, (3) tumors in the bursa of Fabricius, and (4) cytological differences in the tumors. Table 1 gives some diagnostic guidelines.

TABLE 1.—*Guidelines for differential diagnosis of LL and MD tumors*¹

Variable	Lymphoid leukosis	Marek's disease
Age of occurrence	16 weeks or older	6 weeks or older.
Neural signs and lesions ..	No	Yes.
Tumor location:		
Bursa of Fabricius ..	Yes	No.
Visceral organs	Yes	Yes.
Eye	No	Yes.
Skin	No	Yes.
Muscle	No	Yes.
Cell type	Lymphoblasts	Small, medium, and large lymphocytes; few lymphoblasts and plasma cells.

¹ Both types of tumors have been observed in same flock.

Age of Occurrence

Mortality from LL may start as early as about 16 weeks of age. Major losses usually occur at about 26 weeks and peak between 24 and 36 weeks.

Until recently it was thought that MD occurred only in birds less than 16 weeks of age and that all visceral leukotic lesions after 16 weeks were due to LL. It is now recognized that MD may occur at any age after about 6 weeks but most frequently between 8 and 24 weeks. Generally neural lesions are seen in the initial stages of an MD outbreak and visceral lesions appear later. However, this is variable and neural involvement can occur at any age.

For birds with visceral tumors and without neural lesions, LL can be ruled out if the birds are less than 16 weeks of age. If tumors occur after this age, differential diagnosis must largely be based on microscopic examination of tumor smears or sections (figs. 8, 9, 11, 12; pl. 1, A and B).

Neural Signs and Lesions

Neural involvement is commonly seen in MD and is strikingly absent in LL. Although such neural involvement is usually manifested by clinical signs, such as paresis, paralysis, or depression, tumors may occur in clinically normal birds and may be observed at postmortem (figs. 13 and 14) or by microscopic examination of tissues (fig. 15). Since neural lesions are often localized and minimal, they can easily be overlooked. Marked variation in their occurrence and size is common; however, when present they are considered specific for MD.

Involvement of Bursa of Fabricius

Normal Appearance

The bursa of Fabricius is located just above the cloaca of the chicken. It is present at hatching and grows rapidly the first 2-3 months, then it normally atrophies and becomes nonfunctional.



FIGURE 13.—Chicken with MD, showing massive tumor of ovary and of right brachial plexus. BN-35269



BN-35260

FIGURE 14.—Lumbar area of chicken with MD, showing tumor involvement of right lumbar and sciatic plexuses.

The regression occurs earlier in males than in females and usually is nearly complete by the time the birds reach sexual maturity. An unknown factor and lymphoid cells that migrate from the bursa to other organs give origin to the follicles, which have a major role in antibody formation in response to antigenic stimulation.

Grossly the bursa of Fabricius is spherical and the inner layer is made of folds or plicae (fig. 16). Each fold consists of numerous follicles with a distinct cortex and medulla, and there is a capillary bed at the corticomedullary junction (figs. 17 and 18).

As normal regression or involution begins, the number and size of bursa follicles and the number of lymphoid cells decrease, the cortex narrows, follicles become cystic, and the medullary framework disappears (fig. 19). As regression proceeds, the epithelial and connective tissues increase.

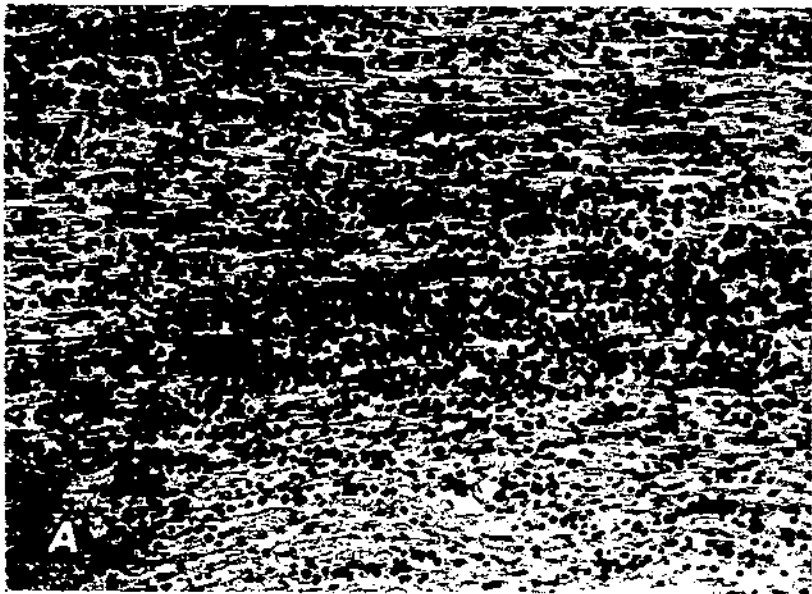


FIGURE 15.—Nerve with MD lesions; A, A type; B, B type. $\times 100$. (Courtesy of H. G. Purchase and P. M. Biggs.)

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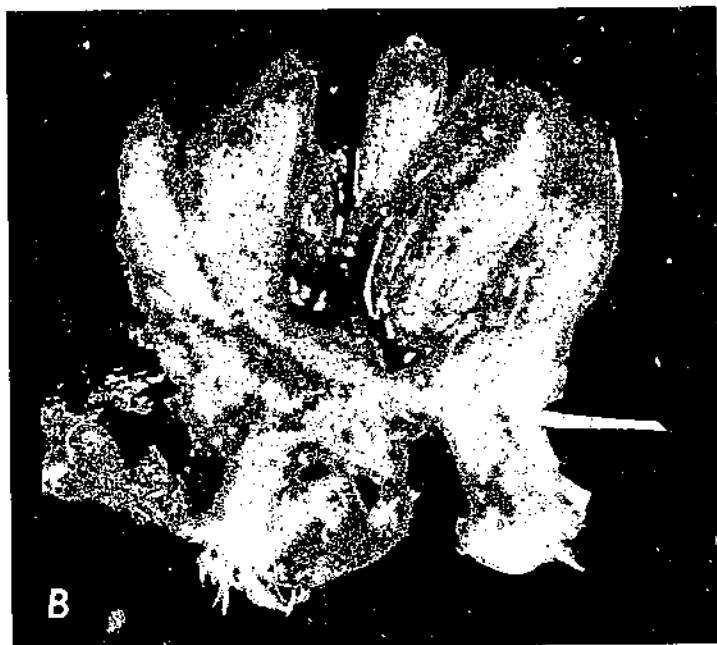
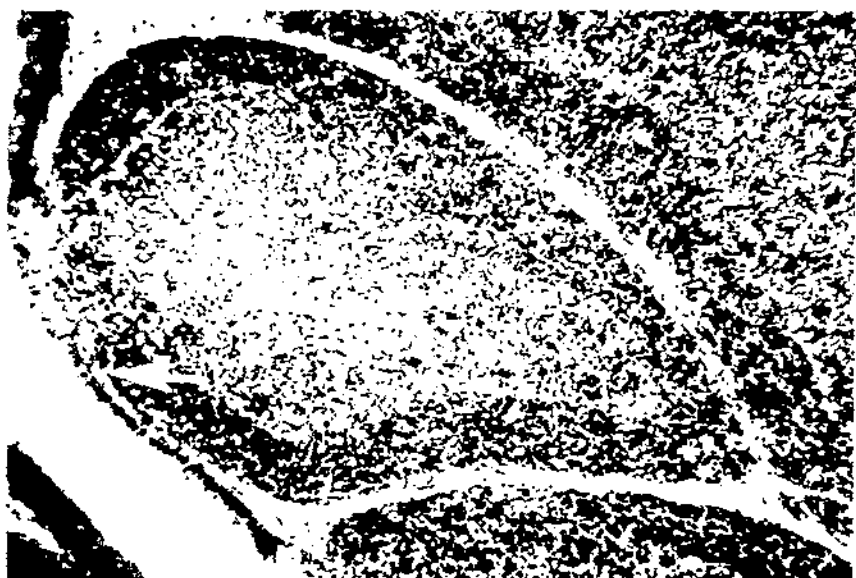


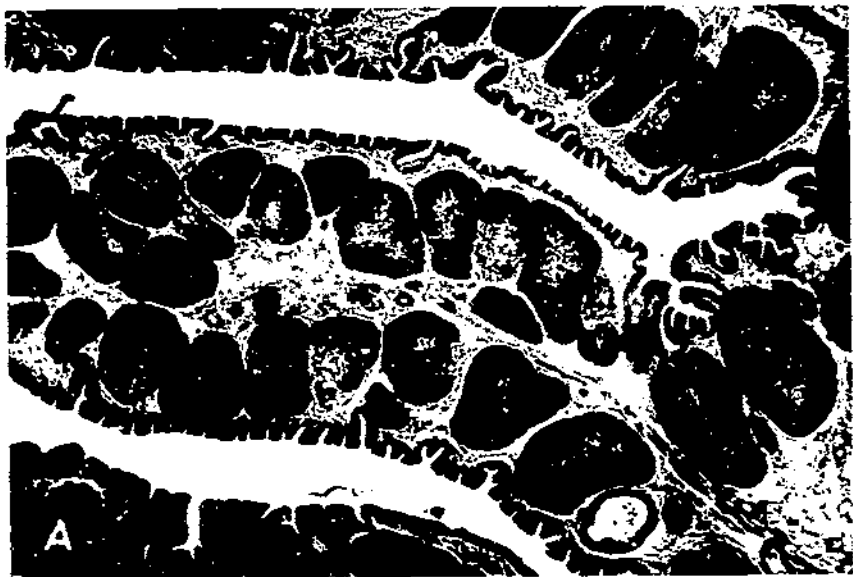
FIGURE 16.—Normal bursa of Fabricius of young chicken: *A*, Attached to cloaca; *B*, dissected and showing normal plicae (folds). BN-35263, BN-35264



BN 35265
 FIGURE 17. Section of plica of normal functional bursa of Fabricius in 5 week-old chicken. $\times 25$.



BN 35266
 FIGURE 18. Single follicle of bursa of Fabricius. Note outer cortex, inner medulla, and continuation of corticomedullary junction (arrow) with gut epithelium. $\times 100$.



BN-35267, BN-35268

FIGURE 19.—A, Histological appearance of plica in normal regressing bursa of Fabricius in 20-week-old pullet, showing decreased number, size, and cell content of follicles ($\times 25$); B, higher magnification shows cystic follicle, epithelization of medullary reticular network, and abundance of sparse connective-tissue network between follicles ($\times 100$).

In Lymphoid Leukosis

A focal or nodular tumor in the bursa of Fabricius in birds 16 weeks and older is considered pathognomonic for LL (fig. 2). LL starts with one or more tumors in the bursa. Grossly observable LL tumors of the bursa do not appear until the bird approaches sexual maturity. Prior to this, tumors can be seen only with the aid of a microscope (fig. 3).

LL tumors of the bursa of Fabricius are nodular and vary in size from a millet seed to a tennis ball. They occur grossly as a single or multiple mass. When multiple masses become large, they usually coalesce.

In Marek's Disease

The bursa of Fabricius generally is smaller than normal for any given age of chicken with MD (fig. 20). This is due to a decrease in the number and cell content of follicles. Also, there may be a slight to moderate lymphocytic proliferation between the follicles. However, in some birds with MD, the bursa is enlarged because of thickening of the walls. This is due to an infiltration of lymphocytes between the follicles, which may become cystic (fig. 21).

Cytological Differences in LL and MD Tumors

LL tumors are composed of a homogeneous population of lymphoblasts (figs. 8 and 9; pl. 1, *A*). In contrast, the MD tumors contain lymphoid cells that vary in maturity from the small lymphocyte to the large lymphoblast (figs. 11 and 12; pl. 1, *B*). Other cells, such as the plasma cell, may also be present in MD tumors.

Methyl green-pyronine staining of fixed tissue sections and tumor smears has helped in differentiating LL and MD tumors. Since the cytoplasm of rapidly dividing lymphoblasts is pyroninophilic and stains a brilliant red, LL tumors, which are made up almost entirely of these cells, show much red staining (pl. 1, *A*). In contrast, MD tumors have only a few lymphoblasts and red staining is proportionately less (pl. 1, *B*).

Examination of stained smears of tumor tissue aid in diagnosis. Their usefulness depends largely on the quality of the smear. For best results, (1) obtain a fresh specimen, (2) select tissue that is all or mostly all tumor, (3) prepare the smear so that the cells are evenly spread and moderately spaced, (4) fix the smear immediately while still wet, and (5) use special cen stains, such as methyl green pyronine or hematoxylin. Suggested procedures are given in the Appendix.

Smears have several advantages over tissue sections since they (1) are rapidly and easily prepared, (2) require few reagents and no special apparatus, (3) yield a clear definition of cell morphology and staining reactions, and (4) can be easily examined at high magnification.

Smears of LL tumors will present a homogeneous population of lymphoblasts similar both in morphological details and in staining characteristics. When the smear is stained with methyl green



FIGURE 20.—Atrophied bursa of Fabricius in Marek's disease. BN-35283



FIGURE 21.—MD-affected bursa plica, showing diffuse severe changes. Note decrease in size of follicles, a cystic follicle, marked proliferation of interfollicular connective tissue, and diffuse infiltration with large numbers of lymphocytes. Bursa with such changes may have thicker than normal plicae. $\times 25$. BN-35284

pyronine, the wide band of cytoplasm stains bright red. When the proportion of lymphoblasts is moderate to high, the smear even at low magnification will appear distinctly red (pl. 1, A).

The color reactions of the methyl green-pyronine stain when optimal show dramatically the differences between the rapidly dividing lymphoblasts and the more mature cells. However, this stain is subject to much chromatic variation, which is due to such factors as the necrobiotic state of the tumor, smear preparation, fixation, and duration of various stages in the staining process.

Another recommended stain for wet-fixed smears is hematoxylin. It is excellent for showing differences in cytologic detail of the nucleus. Staining reactions are not subject to as much variation as with methyl green pyronine; however, there is no differential staining of the cytoplasm.

The type cell in LL tumors is considered to be a lymphoblast because it has much cytoplasm, lightly staining nuclear chromatin, and a large, prominent nucleolus that is either central or eccentric in position.

In smears of MD tumors one sees a pleomorphic population of cells, varying in size, morphologic detail, and staining characteristics. The cell population is made up of small, medium, and large lymphocytes. In addition, some lymphoblasts are present and sometimes plasma cells or myelocytes. The more mature lymphoid cells of MD are different from lymphoblasts in that they have less cytoplasm, more dense, thickly clumped chromatin, and much less prominent or absent nucleolus.

Differences in LL and MD tumor cells are summarized as follows:

	LL	MD
Uniformity	Homogeneous	Pleomorphic.
Size	Large (some variation).	Vary from small to large.
Cytoplasm	Much	Moderate to scant.
Chromatin	Fine network	Dense clumps.
Nucleolus	Prominent	Small or absent.

In organs with diffuse-type lesions, the cells of that organ will also be seen. Thus, a smear of lymphomatous liver may contain many hepatic cells and hepatic cell nuclei, which resemble small lymphocytes. If lymphoid cells are normally present in the organ, i.e., spleen, thymus, and bursa of Fabricius, a differential diagnosis based solely on the smear is very difficult unless the tumor is large or sufficiently focal so that normal tissue can be trimmed away.

DISCUSSION

There is now conclusive evidence for the existence of two distinct lympholeukotic diseases in chickens—lymphoid leukosis and Marek's disease. In both, the lymphoid tumors of the viscera are

similar in gross appearance and location. Until recently almost all diagnoses have been in terms of leukosis or lymphomatosis. These were and still are appropriate for the type of lesions and pathologic processes found. However, the etiologic aspect of any disease is most important. Distinctive terms should be used, and the diagnosis should be directed toward the separation of etiologically distinct diseases.

There are four primary guides for differentiating the two diseases. They are not absolute or infallible but can be used in conjunction with all other data. Thus, clinical signs as well as epidemiologic information when available should be considered.

The needs for specific diagnosis are numerous. Of immediate importance is an assessment of the prevalence and economic importance of these two diseases. It is generally recognized that leukotic lesions occur at a high rate in many flocks and cause great losses due to mortality or condemnation or both. However, no definitive data are available on the rate of occurrence of the two diseases in any area or segment of the poultry industry. When control measures become available, specific diagnoses will be required in order to recommend the appropriate procedures.

SUMMARY

In both lymphoid leukosis (LL) and Marek's disease (MD) of chickens, excessive proliferation and accumulations of lymphoid cells occur. Similarity of the visceral tumors in each disease makes differential diagnosis difficult. However, the following differential features, when considered with other available epizootiologic information, will provide the basis for a specific diagnosis of high reliability.

Age.—LL rarely occurs in chickens prior to 16 weeks of age. MD may occur at any age after about 6 weeks but most frequently between 8 and 24 weeks.

Neural signs and lesions.—Paresis and paralysis are generally not seen in LL, but they are common in MD. Lymphoid accumulations in various parts of the nervous system are common and pathognomonic for MD.

Tumors in bursa of Fabricius.—Focal lymphoid tumors in the bursa of Fabricius are almost always present in LL and never in MD.

Cytology.—LL tumors are composed entirely of lymphoblasts, which are medium to large and have a pyroninophilic cytoplasm, prominent nucleolus, and scanty nuclear chromatin. MD tumors consist of lymphocytes of variable morphology, ranging from predominantly small to large. Some MD tumors have a few lymphoblasts, which are similar in appearance to those in LL tumors. Wet-fixed smears of fresh tumor specimens, stained with cytological and cytochemical stains, are particularly effective in differentiation.

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APPENDIX

Procedures for Preparing and Staining Tumor Smears Specimen Collection and Fixation

Tumors from freshly killed birds are blotted free of blood and gently streaked across previously numbered glass slides until a uniform thin smear is obtained. The slide is then immediately (without drying) immersed in the fixative of choice.

Fixation in 95 percent ethyl alcohol for 1 to 2 minutes gives useful results. For critical study of cellular detail, fixation in Zenker's solution for 18 hours is recommended.

Staining

Methyl Green-Pyronine Stain:

(1) *Alcohol fixed.*—After fixation in 95 percent alcohol for 2 minutes or longer, dip the slide in 70 percent alcohol then in distilled water and blot dry. Immerse in methyl green-pyronine stain for 7 minutes, rinse in distilled water by dipping slowly three times, and gently blot dry. Dehydrate by dipping three to four times in each of three changes of dioxane and finally clear in xylene. Cover slips may be mounted in the usual manner.

(2) *Zenker's fixed.*—First dezenkerize by rinsing the slide in running tapwater for 10 minutes and then place in 80 percent alcohol for 10 minutes and then Gram's iodine for 5 minutes. Again rinse the slide in tapwater, place in 5 percent solution of sodium thiosulfate for 5 minutes, rinse in distilled water, and blot dry. The slide is now ready to stain with methyl green pyronine as previously described.

Hematoxylin Stain:

(1) *Alcohol fixed.*—After alcohol fixation, dip the slide for 5 minutes in 70 percent alcohol and distilled water and then hematoxylin stain for 10 minutes. Rinse in tapwater and then in diluted ammonia water until the stained area is blue, then quickly rinse in distilled water and for 1-minute intervals in 95 percent alcohol, absolute alcohol, and xylene. The slide is ready for mounting after it is dipped a second time in xylene.

(2) *Zenker's fixed.*—Dezenkerize as described under Methyl Green-Pyronine Stain, Zenker's Fixed, and stain as described under Hematoxylin Stain, Alcohol Fixed.

Reagents*Gram's Iodine:*

Potassium iodide	2 gm.
Iodine crystals	1 gm.
Distilled water	300 ml.

Dissolve potassium iodide first, then add iodine.

Hematoxylin Stock Solution:

Hematoxylin	6 gm.
Absolute alcohol	300 ml.
Glacial acetic acid	30 ml.
Glycerin	300 ml.
Distilled water	300 ml.

Dissolve hematoxylin in alcohol and then add glacial acetic acid, glycerin, and water. Add ammonium alum sulfate crystals in excess of saturation point and allow to ripen in sunlight for 2-3 months or longer.

Hematoxylin Working Solution:

Hematoxylin stock solution	1 part
Saturated ammonium alum sulfate	2 parts
Distilled water	2 parts

*Methyl Green-Pyronine Staining Solution:*¹

Methyl green, C.I. 685	1 gm.
Pyronine Y, C.I. 739	0.15 gm.
Distilled water	100 ml.

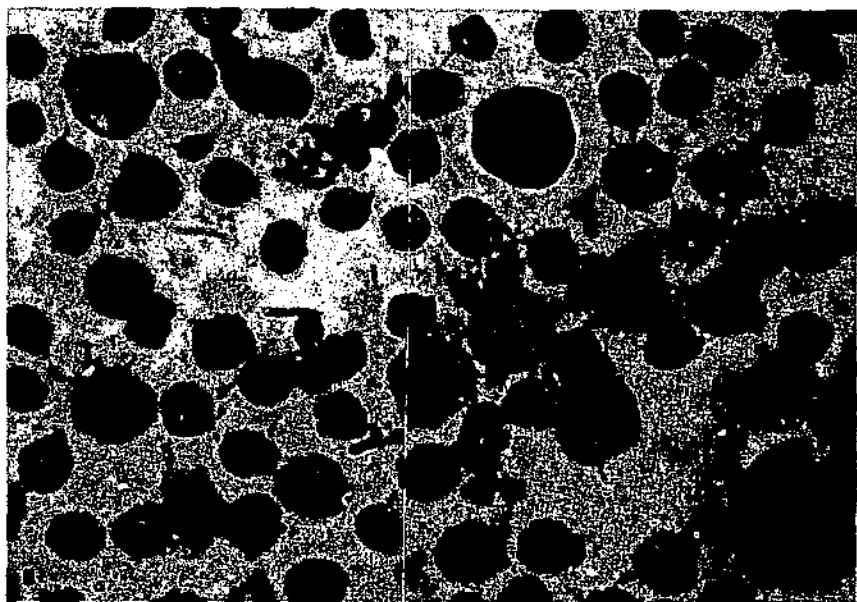
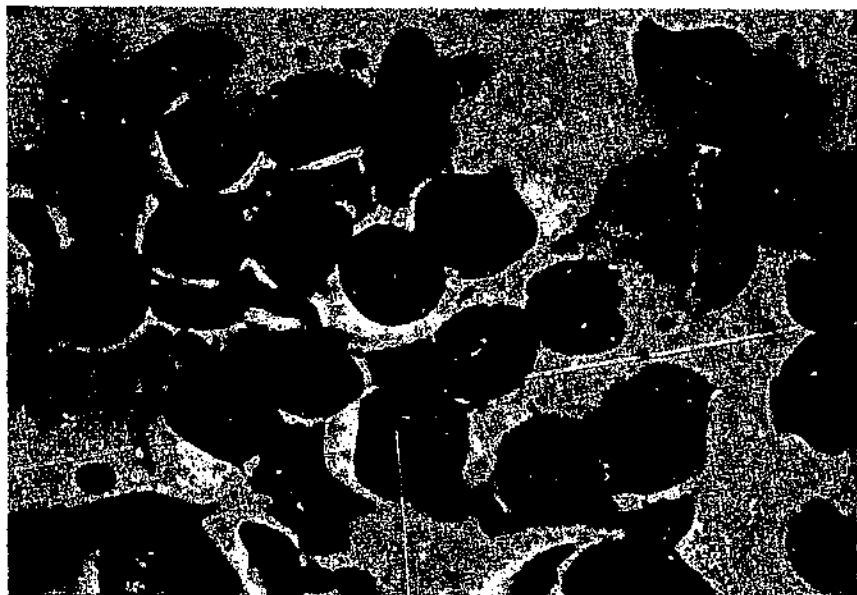
Place dyes in flask and add hot distilled water. Agitate at intervals, incubate for 2 days at room temperature, and then filter the solution.

Zenker's Stock:

Potassium dichromate	2.5 gm.
Mercuric chloride	5 gm.
Distilled water	100 ml.

Add 5 cc. of glacial acetic acid to 100 cc. of Zenker's stock just before use.

¹OPSTAD, A. M. METHYL GREEN-PYRONINE STAIN FOR PLASMA CELLS IN TISSUES. Stain Technol. 34: 293. 1959.



A, Wet-fixed smear of LL liver tumor stained with methyl green pyronine. Note cell homogeneity, marked pyroninophilia (redness) of cytoplasm, and prominent nucleolus of all lymphoblasts. $\times 2,000$. B, Wet-fixed smear of MD ovary tumor stained with methyl green pyronine. Note cell heterogeneity, sparseness of pyroninophilia of cytoplasm, and lack of pyroninophilic nucleolus in most cells. $\times 2,000$.

END