# PROTEIN CONCENTRATION IN NORMAL MOUSE LYMPHOCYTES, THYMOCYTES, AND MOUSE AND HUMAN LEUKEMIC CELLS AS MEASURED BY INTERFERENCE MICROSCOPY*

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ABSTRACT

The apparent protein content of a single spherical lymphocyte or a similar cell, as well as its diameter, can be measured by a modified and stabilized AO-Baker interference microscope which is either fitted with an AO half-shade eyepiece or connected to a photomultiplier.

This paper gives results for the apparent protein content and the diameter of normal mouse lymphocytes and thymocytes; these do not differ significantly from each other. It also gives values for the apparent protein concentration and the diameter of the lymphocytes of mice of the same strain which have developed leukemia or lymphoma; these values are significantly different from those of normal mice. Related data are given for the apparent protein content and for the diameter of normal human lymphocytes and the white cells found in myeloblastic leukemia and in stem cell leukemia in man; here the values differ significantly from those of the normal human lymphocyte. The rule seems to be that the abnormal white cells become more watery and larger as the disease advances.

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A number of properties of white cells can be measured by the AO-Baker interference microscope (model 9). The sensitivity of this instrument is about 2°, or approximately $\frac{1}{200}$ of a wave length. By using a half-shade eyepiece, it is possible to improve the precision of the instrument five to ten times, and almost the same precision can be obtained by attaching the interference microscope to a suitable photomultiplier, although it is true that the human eye can detect differences smaller than can be detected by most photometers. Considerable instrumentation is necessary in order to obtain this increased precision. The instrumentation will be described first. This will be followed by a description of the method of making the preparations, by a description of how the interference microscope can be used with a photomultiplier, and of how it can be used with a half-shade eyepiece. Finally, the protein concentration in normal lymphocytes and thymocytes of the mouse will be given, and this will be fol-

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lowed by some values for the protein concentration in the leukemic cells of the mouse and of man.

The method depends on there being a relation between the refractive index of a cell of known thickness and its content of proteins and, to a very much smaller extent because of their smaller concentration, of other substances which have a refractive index greater than that of the medium in which the cell is immersed. The way in which the specific refractive increment $\alpha$ varies with the concentration of proteins and other substances has been extensively studied (Adair and Robinson, 1930; Craig and Schmidt, 1932; Perlmann and Longsworth, 1948; and Adair, 1955). For human proteins, it varies between 0.00171 and 0.00183 with a mean value of 0.00180. Extensive values are given by Barer in a reference book (Mellors, 1955; 171 references), and the relations which lead to Equation 1 are discussed at length both by Barer in Mellors' reference book, by Barer (1955), and by Richards (1957; 100 references). In the case of the types of cell considered in this paper, it is probably justifiable to regard the cell as entirely composed of protein in solution, certainly as a first approximation.[1]

The method is reliable to within 10 per cent for the estimation of Hb in normal human red cells, the discrepancy being due to the presence of substances in low concentration which have an $\alpha$-value of their own (Ponder, 1958). A small discrepancy (about 5 per cent) no doubt exists in the case of lymphocytes, thymocytes, etc.; hence the method is said to determine "apparent protein" (see below).

*1. Instrumentation.*—The AO-Baker interference microscope, model 9, is mounted on a board 22 inches long, 15 inches wide, and 1 inch thick; this is done by recessing the board by ¼ inch in such a way as to contain the base of the microscope. An American Optical Company lamp with a circular base is recessed in a similar manner so that the center of the base is about 11 inches from the center of the mirror and the light path of the microscope. Both the lamp and the microscope are further secured by metal brackets held to the board by screws. The distances between the light

[1] The question may be asked whether the determination of $C_i$ in Equation 1 is valid; *i.e.*, whether a protein determination from refractive index measurements agrees with determinations of protein by an independent method such as the measurement of dry weight. There is no way of measuring protein concentration in single lymphocytes, etc., but the mean protein concentration as found with the interference microscope ought to agree with the mean protein concentration found by an analytical method applied to a mass of cells. It has been shown by a number of investigators (in particular, see Perlmann and Longsworth, 1948) that the agreement is very good. If the agreement were poor or bad, the Tiselius electrophoresis method would be invalid. Unfortunately, there is only one value for the protein content of the lymphocyte (Enders and Herget, 1929), but this value differs from the value calculated from measurements with the interference microscope by less than 5 per cent.

source in the arc and the mirror of the microscope with its light path are such that Köhler illumination is easily obtained. The board itself rests on a number of rubber mats which should not be too resilient.

The parts of the microscope body which lie immediately above the plane of the analyzer are replaced by a double tube with a beam splitter[2] which gives (*a*) a vertical light path and (*b*) a light path at an angle of 45° in the same vertical plane as the light path (*a*), the light path below the analyzer, and the illuminating beam which falls from the lamp on the mirror of the microscope. The vertical light path (*a*) of this device carries 75 per cent of the light, and may either terminate in a half-shade eyepiece or may contain an eyepiece (30×) which has within it a metal plate perforated by a central 1 mm. hole placed so that the hole is in focus in the front lens of the eyepiece. The search unit of a photomultiplier (Photovolt Corporation, line operated model 520-M) can be attached to this eyepiece and the tube surrounding it in a light-proof manner and is connected to the photomultiplier; the latter must work at its highest degree of photomultiplication (at least $10^5$ times).

The second light path (*b*), situated at 45° from the first, carries a filar micrometer, 16 divisions of which represent 1 $\mu$. This magnification results from the length of the 45° light path and from the characteristics of the micrometer; it should not be reduced and might even be increased. This 45° light path carries 25 per cent of the incident light.

The microscope is stabilized by two cylinders of brass filled with lead, each 2 inches in diameter and 14 inches high,[3] mounted on the 22 by 15 inch board. Lines drawn through the centers of these cylinders would pass through the center of the objective and also through the center of the vertical light path (*a*) above the analyzer. The centers of the cylinders are about 6 inches to the left and to the right of the objective. The tops of the cylinders are linked together by a flat strip of metal 14 inches long, 2 inches wide, and ¼ inch thick. In the center of this piece of metal, *i.e.*, where it would lie over the vertical light path, a circular aperture is cut and a collar is fitted into the aperture so that its inner diameter is almost exactly that of the outer diameter of the tube through which the vertical light path (*a*) passes. This collar can be clamped to the outside of the tube through which the vertical light path (*a*) passes by turning a horizontally projecting screw. When this screw is turned so as to tighten the collar, the vertical light path of the microscope, from the objective below to the top of the tube through which the vertical light path passes, is rendered immobile. Lateral motion of the tubes containing the vertical light path (*a*) and the inclined light path (*b*) is prevented by the mass of the two lead cylinders and the piece of metal which joins them.

*2. Preparations.*—Thymus and lymph glands of mice (not always easily distinguishable when the glands are in the neck) are removed and cut a few times with scissors. The cut material is dipped in a small amount of the animal's own serum so that a few cells become detached and suspended. The dilution must be large so that any field of the interference microscope contains only a few cells (about 5). A

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[2] Specially constructed for me by the American Optical Company.

[3] The interference microscope was stabilized as described by Mr. Paul Cutajar of the New York University Machine Shop, to whom my thanks are due.

small drop of the suspension is made between a slide and a cover glass which is lightly vaselined along its edges, care being taken to exclude air bubbles, dust particles, etc. The same method is used in the case of the lymphocytes of human lymph glands.

The preparations of human white cells from the blood depend on their number and their nature. If the white cell count is high, the blood collected in heparin or preferably disodium sequestrin is allowed to sediment. The plasma and the buffy coat are removed and are centrifuged gently in a hematocrit tube. Most of the plasma is removed and set aside; the remaining plasma, together with the upper part of the buffy coat which contains most of the platelets, is discarded. The remainder of the buffy coat, which contains white cells, is added to the plasma set aside, so as to give a suspension which shows only a few white cells per field of the interference microscope. A vaseline-sealed preparation is made. When the white cell count is low, the procedure is much more difficult, but it is essentially the same. It does not matter whether the white cells are normal lymphocytes, the lymphocytes of lymphatic leukemia, or even stem cells; it is necessary, however, that they be spherical, as they usually are in these preparations. It is essential that the material should be as fresh as possible.

We have had little success with many of the proposed separation methods, such as flotation in albumin or dextran.

*3. Protein Measured by the Interference Microscope and a Photomultiplier.*—The eyepiece (30×) with the metal plate perforated by a 1 mm. hole is placed in the upper end of the vertical light path (*a*). There is one position of the cross-hairs of the filar micrometer in the second light path (*b*) which corresponds to the center of the 1 mm. hole in the eyepiece in the vertical light path(*a*). This position must be found by trial.

The cross-hairs of the filar micrometer in the light path(*b*) are set in this predetermined position, and a cell is brought with its center over the intersection of the cross-hairs. This is easily done if the conventional moving stage supplied with model 9 is replaced by an AO microglide stage (Catalogue No. 1547) which consists of an upper circular plate which is moved by hand over a lower plate, the two plates being separated by a layer of heavy grease. When viewed in the eyepiece in the vertical light path (*a*), the cell will be seen to fill the 1 mm. hole. The search unit of the photomultiplier is then put in place above the eyepiece and is screwed to the tube of the vertical light path; this makes a light-proof connection. The photomultiplier is turned to its highest degree of photomultiplication and the needle of its galvanometer is brought to some suitable value for density such as 0.7. Using monochromatic green light and with the interference microscope adjusted in the usual way, an expanded first order fringe filling the back aperture of the objective as seen with a phase telescope, the analyzer is slowly turned until the galvanometer needle of the photomultiplier passes through a point of maximum density. The reading in degrees of the analyzer is noted. The cell can then be moved a short distance from the intersection of the cross-hairs; the surround then fills the 1 mm. hole in the eyepiece and the analyzer is moved until the galvanometer again passes through a point of maximum density, the position of which is noted. The first reading of the analyzer gives the position which illuminates the cell least, whereas the second reading gives the position at which the surround is least illuminated. The diameter of the cell is measured with the calibrated filar micrometer in light path (*b*).

*4. The Interference Microscope Used with a Half-Shade Eyepiece.*—The microscope

is adjusted in the usual way by filling the back of the objective with an expanded fringe as seen through a phase telescope. The American Optical Company half-shade eyepiece is inserted at the top of the vertical light path (*a*) and held in position by its retaining screw. A cell is brought to such a position that the dividing line seen in the image of the half-shade eyepiece bisects it. This is easily done if the conventional moving stage has been replaced by an AO microglide stage. The analyzer of the half-shade eyepiece (the analyzer in the body of the interference microscope being turned to the "out" position) is turned so that both sides of the bisected cell are equally dark. A reading of the position of the analyzer is made. The analyzer is then turned so that both sides of the neighboring surround, which is also bisected by the vertical line, are equally dark. Another reading of the position of the analyzer is made and the difference between this and the previous reading is 0.5 $\theta$. The diameter of the cell is measured with the filar micrometer in light path (*b*).

Whether the photomultiplier or the half-shade eyepiece is used, the concentration of "apparent protein" as a percentage is

$$C_i = \frac{0.84 \cdot \theta}{t} \tag{1}$$

in which $t$ is the thickness of the spherical cell. In this expression, $C_i$ is called the "apparent protein" because the interference microscope under the conditions used here measures the sum of the concentrations of all the substances in the cell which have a refractive index greater than that of the medium in which the cell is suspended.

## RESULTS

The results (diameter $t$ and apparent protein concentration $C_i$ as a percentage) for the lymphocytes from glands of 10 normal mice (AKR strain) and the thymocytes of 10 normal mice are given in Table I. There is no significant difference as regards either $t$ or $C_i$ between the lymphocytes from normal glands and the thymocytes of normal mice.

The situation is different in the case of lymphocytes from the glands of normal mice and lymphocytes from the glands of mice which have developed leukemia (AKR strain, better referred to as spontaneous mouse lymphoma than as mouse leukemia). In the leukemic or lymphomatous mouse, $C_i$ is smaller than $C_i$ in the normal mouse, while $t$ is larger in the leukemic or lymphomatous mouse. The difference in the means is 2.8 and 2.0 times the standard error of the difference of the means.

Some results for man and leukemia in man are shown in Table II. The purpose of adding them is to show that the method is of general utility. In myeloblastic leukemia in man, $C_i$ is smaller than it is for the normal lymphocyte;[4]

[4] It is doubtful whether normal human lymphocytes should be compared with myeloblasts or stem cells, but normal human myeloblasts and stem cells are virtually unobtainable. The purpose of the comparison, at all events, is to show that the diameter and apparent protein concentration of the leukemic myeloblast and stem cell differ greatly from those of the normal lymphocyte.

TABLE I

| Normal mouse lymphocytes | | Normal mouse thymocytes | |
|---|---|---|---|
| $t$ | $C_t$ | $t$ | $C_t$ |
| 7.3 ± 1.13* | 15.3 ± 2.60* | 7.0 ± 1.32* | 14.1 ± 3.41* |
| 6.9 ± 0.68 | 11.9 ± 3.41 | 8.3 ± 1.19 | 16.2 ± 2.91 |
| 7.3 ± 1.10 | 14.7 ± 1.60 | 7.8 ± 0.65 | 11.8 ± 1.54 |
| 6.4 ± 0.91 | 14.3 ± 1.88 | 7.3 ± 1.72 | 13.0 ± 3.61 |
| 6.8 ± 1.19 | 15.4 ± 2.10 | 6.4 ± 1.24 | 14.4 ± 3.11 |
| 6.6 ± 0.67 | 15.1 ± 3.48 | 6.6 ± 0.60 | 13.3 ± 2.18 |
| 6.5 ± 0.62 | 12.7 ± 2.14 | 7.4 ± 1.20 | 13.3 ± 1.30 |
| 7.3 ± 0.81 | 12.1 ± 1.65 | 7.3 ± 1.02 | 13.1 ± 2.26 |
| 6.5 ± 0.79 | 12.2 ± 1.91 | 7.3 ± 0.97 | 11.2 ± 2.87 |
| 6.5 ± 0.70 | 15.2 ± 2.68 | 8.8 ± 0.77 | 10.9 ± 0.75 |
| 6.8 ± 0.28‡ | 13.9 ± 0.78‡ | 7.4 ± 0.36‡ | 13.1 ± 0.80‡ |

* The figures following the mean are the standard deviations of the measurements in the individual case.

‡ These are the standard errors of the mean.

The same remarks apply to Table II.

TABLE II

| | $t$ | $C_t$ |
|---|---|---|
| Lymphocytes, mouse leukemia | 7.4 ± 0.49 | 10.7 ± 1.62 |
| | 6.9 ± 0.70 | 9.2 ± 5.11 |
| | 6.8 ± 1.25 | 15.1 ± 2.70 |
| | 8.8 ± 1.19 | 9.7 ± 2.32 |
| | 10.4 ± 3.30 | 14.6 ± 3.23 |
| | 9.1 ± 2.01 | 8.8 ± 3.72 |
| | 8.6 ± 0.94 | 11.5 ± 2.02 |
| | 8.3 ± 1.41 | 11.4 ± 2.96 |
| Human, normal lymphocytes (average of 10 persons) | 6.3 ± 0.61 | 14.1 ± 1.79 |
| Human leukemia, myeloblastic | 9.6 ± 1.03 | 10.4 ± 3.08 |
| | 9.5 ± 1.11 | 5.5 ± 2.96 |
| | 7.5 ± 0.38 | 9.5 ± 1.91 |
| | 9.3 ± 2.71 | 9.5 ± 3.90 |
| | 8.0 ± 1.40 | 11.8 ± 3.30 |
| | 8.8 ± 1.33 | 9.3 ± 3.30 |
| Human stem cell leukemia | 7.7 ± 0.76 | 10.2 ± 4.15 |
| | 10.7 ± 3.20 | 11.4 ± 4.00 |
| | 8.0 ± 0.39 | 9.4 ± 2.90 |
| | 14.6 ± 1.61 | 6.8 ± 3.71 |
| | 13.8 ± 2.55 | 6.1 ± 2.88 |
| | 11.0 ± 1.70 | 8.8 ± 3.53 |

the difference is significant. The value of $t$, on the other hand, is greater in myeloblastic leukemia than it is in the normal person; again the difference is significant. The same thing is true in stem cell leukemia in man; $C_t$ is significantly less than in the normal, and $t$ is significantly greater. In these relatively acute leukemias, the rule seems to be that the affected white cells become larger and more watery; *i.e.*, contain less apparent protein. If a single case is followed by repeated observations, the size of the affected white cells almost invariably increases and the apparent protein decreases as the disease advances.

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