References and Notes

1. W. Kauzmann, *Advan. Protein Chem.* **14**, 1 (1959).
2. S. J. Singer, *ibid.* **17**, 1 (1962).
3. A. M. Michelson, *Chemistry of Nucleosides and Nucleotides* (Academic Press, New York, 1963).
4. T. T. Herskovits, S. J. Singer, E. P. Geiduschek, *Arch. Biochem. Biophys.* **94**, 99 (1961).
5. T. T. Herskovits, *ibid.* **97**, 474 (1962).
6. ———, *Biochemistry* **2**, 335 (1963).
7. J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, V. C. Shore, *Nature* **190**, 666 (1961).
8. D. R. Davies, *Annu. Rev. Biochem.* **36**, 646 (1967).
9. G. Nemethy and H. A. Scheraga, *J. Phys. Chem.* **66**, 1773 (1962).
10. T. T. Herskovits and H. Jaillet, *Fed. Proc.* **27**, 771 (1968).
11. S. N. Timasheff and M. J. Gorbunoff, *Annu. Rev. Biochem.* **36**, 13 (1967).
12. B. Chance, R. W. Esterbrook, T. Yonetani, Eds., *Hemes and Hemoproteins* (Academic Press, New York, 1966).
13. T. T. Herskovits, in *Methods of Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1967), vol. 11, p. 748.
14. D. W. Urry, *J. Biol. Chem.* **242**, 4441 (1967).
15. P. J. Urnes, K. Imahori, P. Doty, *Proc. Nat. Acad. Sci. U.S.* **47**, 1635 (1961); S. Beychok and E. R. Blout, *J. Mol. Biol.* **3**, 769 (1961).
16. M. Muirhead and M. F. Perutz, *Nature* **199**, 633 (1963).
17. The studies of Urry (*14*) on the absorption and optical rotatory properties of the heme chromophore in the ultraviolet region suggest that changes in the environment of the heme and aromatic chromophores may elicit small changes in the observed optical activity in the polypeptide region (233 nm). These effects, however, must be relatively small, since (i) estimates of the helix content of myoglobin and hemoglobin based on the rotatory dispersion of these proteins in the region from 240 to 360 nm (*15*) are in reasonable agreement with x-ray crystallographic data (*7*, *8*) and (ii) the rotational strength or molar ellipticities in the aromatic region (260 to 280 nm) are at most 2 to 5 percent of the corresponding values of these parameters in the polypeptide region. Deoxygenation of hemoglobin, which is accompanied by an appreciable change in the three-dimensional structure of the protein (*16*), is accompanied by only a relatively small change in the molar ellipticity from about -2.4×10^6 to -2.8×10^6 at 222 nm. The molar ellipticity at 280 to 290 nm actually changes sign [from (4 to 8 $\times 10^3$) to about (-5 to -12×10^3)], yet its influence on the ellipticity at 222 nm is only marginal.
18. C. Tanford, P. K. De, V. G. Taggart, *J. Amer. Chem. Soc.* **82**, 6028 (1960); C. Tanford, C. E. Buckley, P. K. De, E. P. Lively, *J. Biol. Chem.* **237**, 1168 (1962).
19. K. Hamaguchi and E. P. Geiduschek, *J. Amer. Chem. Soc.* **84**, 1329 (1962).
20. P. H. von Hippel and K.-Y. Wong, *Science* **145**, 577 (1964); *J. Biol. Chem.* **240**, 3909 (1965).
21. E. E. Schrier and H. A. Scheraga, *Biochim. Biophys. Acta* **64**, 406 (1962).
22. K. Hamaguchi and H. Sakai, *J. Biochem. Tokyo* **57**, 721 (1965).
23. L. Levine, J. A. Gordon, W. P. Jencks, *Biochemistry* **2**, 168 (1963).
24. J. C. Kendrew, *Brookhaven Symp. Biol.* **15**, 216 (1962).
25. Y. Nozaki and C. Tanford, *J. Biol. Chem.* **238**, 4074 (1963).
26. ———, *ibid.* **240**, 3568 (1965).
27. J. F. Brandts, *J. Amer. Chem. Soc.* **86**, 4291 (1964).
28. D. R. Robinson and W. P. Jencks, *ibid.* **87**, 2462 (1965).
29. J. A. Gordon and P. Jencks, *Biochemistry* **2**, 47 (1963).
30. H. S. Frank and M. W. Evans, *J. Phys. Chem.* **13**, 507 (1945).

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Cytotoxic Test Automation: A Live-Dead Cell Differential Counter

Abstract. *An appropriately modified cell spectrophotometer was used successfully for performing automatic counts of live and dead cells in the cytotoxic test, with trypan blue staining as an indicator of dead cells and light scattering to identify viable cells.*

The cytotoxic test of Gorer and O'Gorman (*1*, *2*) in which viable cells are killed on exposure to antibody and complement is invaluable in the study of transplantation antigens in man (*3*) as well as in animals. Various objective criteria of cell death have been used successfully for this test, based on visible changes in the cell with or without the aid of dyes, or on cytolysis as in the ^{51}Cr release test (*4*). A limitation of the prior methods is the need for visual counting. However it is now feasible to perform counts of live and dead cells automatically, with a new cell spectrophotometer (*5*, *6*) modified for this purpose.

Theoretically, any difference between living and dead cells that can be distinguished visually could serve for automatic counting by cell spectrophotometry. It is simplest to identify dead cells by staining with trypan blue, and unstained living cells by their scattering of light. Trypan blue is already widely used for visual counts, and it is generally recognized as a valid indicator of cell death.

The fluorescein diacetate technique (*7*) for identifying living cells at first appeared to be eminently suitable for automatic spectrophotometry. Adequate emission signals were obtained from fluorescein-containing living cells, whereas we could identify dead cells by scattering of light. Alternatively, in experiments with suspensions of living and heat-killed cells, we found that fluorescein diacetate and trypan blue could be used together, the dead cells then being identified by light absorption due to trypan blue staining (*8*). However, if the cells are killed by antibody, as in the cytotoxic test, they must be washed before the addition of fluorescein diacetate to prevent fluorescein release by the esterase present in serum. This introduces an extra step after the incubation, which is undesirable.

The procedure finally chosen was as follows. For the cytotoxic test (*2*), doubling dilutions of isoantiserum of 0.1 ml volumes were dispensed in 2-ml tubes. To each tube 0.1 ml of cell suspension (5×10^6 cell/ml) was then added, followed by 0.1 ml of fresh guinea pig serum, diluted 1:3, as the source of complement. Medium "199" was the diluent and suspending medium.

After incubation at 37°C, with occasional shaking for 45 minutes, the tubes were placed on ice. Immediately before examination, 0.15 ml of freshly prepared 0.24 percent trypan blue in 0.85 percent NaCl was added to each tube, to stain the dead cells. A final suspension in the range of 1×10^6, or less, was necessary to minimize coincident passages of cells through the channel. When the original cell suspension is more concentrated, the volume of trypan blue should be increased to obtain a final cell suspension suitable for counting.

An added precaution particularly necessary when small cells are counted is the removal of particulate matter from the trypan blue solution, serums, and Medium "199." This is done by filtration through Millipore (R) (1.25 μ) filter.

The automatic spectrophotometer previously described (*5*) was used with a

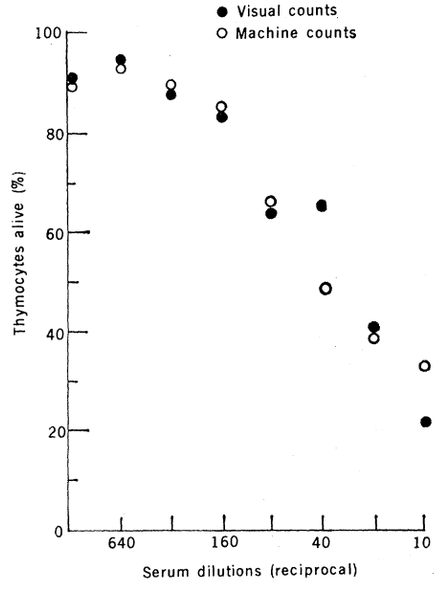

Fig. 1. Cytotoxic test on mouse thymocytes with H-2 isoantiserum showing close similarity of visual and automatic counts. Reagents were deliberately not filtered on this occasion in order to show the error due to particulate matter that scatters sufficient light to generate signals adding to the live cell count. As the proportion of dead cells increases, there is a relative increase in the error that becomes apparent by the shape of the curve, as well as by comparison with visual counts at a serum dilution of 1 : 10.

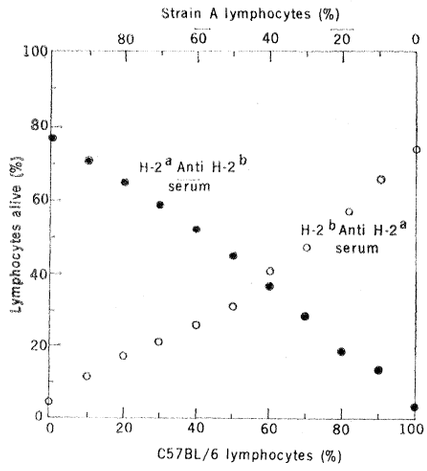

Fig. 2. Cytotoxic tests with H-2 antiserums on C57BL/6 mouse lymphocytes and on A-strain mouse lymphocyte suspensions, mixed in various proportions by volume. The slight curvature of the two plots and their intersection near 60 percent (abscissa) rather than at 50 percent was predictable from an inequality of the cell counts of the original suspensions. The counts are highly reproducible, and the test illustrated here better demonstrates their accuracy than does a comparison with visual counts. Serum and reagents were filtered for this test.

modified dark-field optical system (*9*).

The absorption at 5900 Å by dead cells stained with trypan blue was greater than their scatter and caused a decrease in total light through the optical system. Living cells scattered the incident energy without significant absorption and caused an increase in the light transmitted through the optical system. Thus, the photomultiplier signals due to dead cells and living cells were of opposite polarity. The two pulse trains produced were counted on independent scalers. The scaler values were used directly as counts of live and dead cells in a sample. For most samples at least 5000 cells were counted at a rate of about 200 cells/sec.

Channels with 100-μ and 25-μ constrictions were also tested. Better signals were obtained with the smaller channel, particularly with small cells, but clogging of the channel was more frequent. This was not a difficulty with the 50-μ channel.

The accuracy of this automated method of counting was studied with a variety of cells and compared with visual counts. For the most part H-2 isoantiserums were reacted with mouse lymphocytes and thymocytes, EL4 ascites leukemia cells, and Meth A ascites sarcoma cells. Even with the smallest cells, signals were adequate to distinguish from electrical noise; counts were reproducible within 2 percent and were paralleled with visual counts (Figs. 1 and 2).

There was a systematic error which was especially marked with small cells such as thymocytes (Fig. 1). This arose from particles that scattered sufficient light into the dark field to be registered as living cells. The error was most obtrusive as the number of dead cells approached 100 percent. At that point, machine counts indicated a residue of usually 5 to 10 percent of living cells, due to these particles. The particles which register as live cells are small, and when large cells were being counted the threshold for signals could be adjusted to exclude them; with smaller cells, this was not possible. The error was minimized by filtering the serums, Medium "199," and the trypan blue solution as described above. Corrections also can be made by obtaining background counts on an appropriate volume of serums, Medium "199," and trypan blue without cells, but we found this unnecessary after filtration. Erythrocytes should be excluded as far as possible in preparation of cell suspensions since they refract light without taking up trypan blue, and are counted as living cells.

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References and Notes

1. P. A. Gorer and P. O'Gorman, *Transplant. Bull.* **3**, 142 (1956).
2. E. A. Boyse, J. Old, I. Chouroulinkov, *Methods Med. Res.* **10**, 39 (1964).
3. E. S. Curtoni, P. L. Mattiuz, R. M. Tossi, Eds., *Histocompatibility Testing 1967* (Williams & Wilkins, Baltimore, 1967).
4. A. R. Sanderson, *Immunology* **9**, 287 (1965); H. Wigzell, *Transplantation* **3**, 423 (1965).
5. L. A. Kamentsky, M. R. Melamed, H. Derman, *Science* **150**, 630 (1965).
6. L. A. Kamentsky and M. R. Melamed, *Ann. N.Y. Acad. Sci.*, in press.
7. F. Celada and B. Rotman, *Proc. Nat. Acad. Sci. U.S.* **57**, 630 (1967).
8. R. O'Brien and P. Gottlieb, *J. Histochem. Cytochem.*, in press.
9. An Osram XBO-75 xenon source was used with Koehler illumination of a 50-μ constriction in the flow channel. A 2.8-mm, 0.72 N.A. Polaroid Grey (*10*) reflecting objective lens (Bausch and Lomb) imaged the flow channel onto an aperture, limiting the object field to a 50-μ square. The back focal plane of the objective was imaged through a 5900-Å interference filter onto a Fairchild-2485 photomultiplier tube. The aperture of the Carl Zeiss UV-Kond condenser (Ultrafluor; 0.8 N.A. with iris diaphragm) was adjusted so that all but about 25 percent of the entrance cone of light to the objective was obscured by the central mirror of the reflecting system.
10. D. S. Grey, *J. Opt. Soc. Amer.* **40**, 283 (1950).
11. We thank C. Iritani for performing the cytotoxic tests. E.A.B. was supported in part by National Cancer Institute grant No. CA-08748 and a John A. Hartford Foundation, Inc. grant.
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Human C'3: Evidence for the Liver as the Primary Site of Synthesis

Abstract. *The liver is the primary, if not sole, site of synthesis of the third component of human complement, as shown by a change in the recipient from C'3 $FS_{0.6}$ to C'3 SS, the donor type, following homotransplantation of the liver.*

Polymorphism in the plasma proteins has provided useful markers in determining their source in homotransplantation of the human liver. If a given protein is of different type in liver donor and recipient, and if the recipient's type changes to that of the donor after transplantation, the liver must be the primary, if not sole, site of production of that protein. Such a change in type has been demonstrated for haptoglobin (*1*) and more recently for Gc-globulin (*2*). Experiments in animals, which utilize incorporation of radioactive labels (*3*) and fluorescent antibody localization (*4*), have shown that haptoglobin is indeed synthesized by the liver.

With the recognition of allotypy in the third component of human complement (C'3), the question of hepatic synthesis of this protein can be examined in similar fashion. There are seven known alleles for C'3 (*5*) and the various gene products are identified by differences in electrophoretic mobility. In Caucasians, the C'3 S (slow) allele has an approximate gene frequency of 0.75, while the F (fast) gene has a frequency of about 0.25. The other allotypes, F_1, $F_{0.8}$, $F_{0.5}$, $S_{0.6}$, and S_1, are relatively rare.

Typing of C'3 was performed by prolonged agarose electrophoresis of fresh serum or serum promptly separated from clotted blood and stored at −80°C as previously described (*5*); and typing of haptoglobin, by the method of Smithies (*6*) with starch gel electrophoresis and a discontinuous buffer system (*7*). Quantitation of C'3 was carried out with the electroimmunodiffusion technique of Laurell (*5, 8*) or a nephelometric method (*5, 9*) or both,