tion under an applied electric field. Cohen *et al.* have already suggested that a large part of the birefringence change arose in radially oriented molecules associated with the membrane, and they are not different in nature from the dipoles we have so far discussed. From Langevin's theory, an electric field will induce orientational polarizability of polar molecules and thus change the refractive index of the dielectric (*11*). Thus the finding by Cohen *et al.* lends a further support to the dipole theory for nerve excitation.

The dipole flip-flop can offer an immediate explanation for the impulse propagation along an axon. Since all dipoles have the same energy levels, dipole flop from energy level E_2 to E_1 at point A will cause dipole flip from energy level E_1 to E_2 at the neighboring point B by the law of conservation of energy. The barrier potential at B would be lowered and a nerve impulse would appear there. This process will go on and on and one sees pulse propagation along the axon.

The comparison of Fig. 1 with the structure and the potential profile of a *p-n-p* transistor shows similarity. In a transistor, the emitter barrier is lowered by applying a forward bias which draws carriers away from the junction. In an axon, the outer barrier is lowered by applying a cathodic potential which causes dipole flipping. Since the physical principles governing the dynamics of charged particles are the same, there is no reason why the axon would not take a transistor action once its outer barrier is lowered. Such a theory was proposed previously by Wei and it did predict many features of nerve conduction (*12*). However, at that time (1966), there was no evidence for the existence of surface dipoles and hence the exact roles played by surface dipoles were not too clear. Now, in the face of the recent discoveries of axon membrane (physical) properties, the dipole theory appears more plausible and thus may serve as a physical basis for nerve excitation and conduction.

LING Y. WEI
Biophysical Research Laboratory,
Electrical Engineering Department,
University of Waterloo,
Waterloo, Ontario, Canada

References and Notes

1. J. R. Segal, *Biophys. J.* **8**, 470 (1968).
2. L. B. Cohen, R. D. Keynes, B. Hille, *Nature* **218**, 438 (1968).
3. A. Fraser and A. H. Frey, *Biophys. J.* **8**, 731 (1968).
4. F. Brown and J. F. Danielli, in *Cytology and Cell Physiology*, G. B. Bourne, Ed. (Academic Press, New York, 1964), p. 249.
5. L. Y. Wei, *Biophys. J.* **8**, 396 (1968).
6. F. H. Johnson, H. Eyring, M. J. Polissar, *The Kinetic Basis of Molecular Biology* (Wiley, New York, 1954), chaps. 11 and 12.
7. J. L. Kavanau, *Structure and Function in Biological Membranes* (Holden-Day, San Francisco, 1965), vol. 1.
8. E. J. Cohen and J. T. Edsall, *Proteins, Amino Acids and Peptides* (Reinhold, New York, 1943).
9. The use of the values of protein dipole moment is merely for the estimating purpose and does not imply that the electric dipoles are located in the protein covering the membrane surface. At present, to my knowledge, there is no direct evidence to indicate the exact location of electric dipoles. However, this knowledge is not very essential to the present theory which is only concerned with physical principles.
10. B. C. Abbott, J. V. Howarth, J. M. Ritchie, *J. Physiol. (London)* **178**, 368 (1965).
11. C. Kittel, *Introduction to Solid State Physics* (Wiley, New York, 1967), chap. 12.
12. L. Y. Wei, *IEEE Spectrum* **3**, 123 (1966).
13. Supported by the National Research Council of Canada under grant No. A-1252.

5 July 1968; revised 16 October 1968

Structural Stability and Solvent Denaturation of Myoglobin

Abstract. *As judged from the midpoints of the denaturation transition of 31 water-miscible alcohols, ureas, and amides, the effectiveness of these denaturing agents on sperm-whale myoglobin increases with increasing chain length and hydrocarbon content, as expected in view of the disorganization of the hydrophobic interior of this protein. Increase in the hydroxyl content, blocking of the functional amino groups of the ureas and amides by alkyl substitution, and branching of the hydrocarbon portion of the denaturants are of less importance in determining the effectiveness of the denaturants.*

Considerable evidence based on both experimental and theoretical studies has indicated the importance of hydrophobic interactions for altering the native structure of proteins and nucleic acids in solution (*1–3*). Previously we have examined the effects of various structurally related denaturants on the conformational stability of DNA (*4–6*). Fairly detailed three-dimensional structures of sperm-whale myoglobin (*7*) and other proteins (*8*), which indicate the location of the various amino acid side chains, have been obtained by x-ray crystallography. The systematic investigation of the effectiveness of structurally related denaturing agents, such as the alcohols, ureas, and amides, on these proteins should help to elucidate the nature of those forces controlling native structure. Such studies should also lead to better understanding of hydrophobic bonding or hydrophobic interactions (*1–3, 9*). We here report on the denaturation of sperm-whale myoglobin by solvents. A brief communication on this subject has been presented, including similar denaturation studies on cytochrome c and α-chymotrypsinogen (*10*).

The denaturation of proteins and nucleic acids can be readily followed by several techniques. Changes in the optical rotatory dispersion (ORD) of proteins at 233 nm reflect mainly alterations in secondary structure or the breakdown and reformation of the helical organization of proteins upon solvent denaturation (*11*). On the other hand, changes in the direct spectra, as well as in the difference spectra of the heme proteins at 409 nm (*12*) and at 286 to 288 nm (*13*), reflect alterations in the environment of the heme moiety and the hydrophobic tyrosyl and tryptophyl residues, respectively. The changes in mean residue rotation ($[M]_{233}$), molar absorbance (ε_M) at 409 nm, and denaturation difference spectra (expressed as the difference in molar absorbance $\Delta\varepsilon_{288}$; measured at 288 nm with $\Delta\varepsilon$ at 325 nm taken as zero reference) produced by the addition of such denaturants as urea and propanol (Fig. 1), for example, are closely parallel (*14–17*) with essentially similar denaturation midpoints, despite the different physical origins of the measured parameters.

The gradual refolding of proteins in helix-promoting solvents above the transition region were first observed by Tanford and co-workers (*18*) in β-lactoglobulin and γ-globulin. With myoglobin, similar changes are noted in solvents such as propanol (Fig. 1) and other alcohols where the gradual refolding of the polypeptide chain into an altered conformation (*1, 2, 18*) is accompanied by a gradual change in $[M]_{233}$. Changes in helix content are not accompanied by parallel changes in the environments of the heme and aromatic tryptophyl and tyrosyl chromophores, since no corresponding changes are noted in the ε_{409} and $\Delta\varepsilon_{288}$ curves. Data obtained with a random coil-promoting solvent, urea (Fig. 1), indicate no comparable conformational effects.

There is a striking similarity in the transition profile of myoglobin for a related series of denaturing agents, both in their relative magnitudes and in the sharpness or breadth of the transitions. Figure 2 presents a comparison for such a series, the straight-chain alcohols.

Optical rotatory dispersion was measured in a recording spectropolarimeter (Cary 60); direct spectra and difference spectra were measured in a recording spectrophotometer (Cary 14) or in a double-beam instrument (Coleman-Hitachi 124), usually after equilibration for 0.5 to 5 hours at 25° ± 1°C. With most reagents the rate of denaturation of myoglobin was fairly high; thus essentially equilibrium values of the measured parameters could be obtained very shortly after mixing. Measurements of ORD and difference spectra are limited to a few denaturing solvents (such as the alcohols and some of the ureas) that are sufficiently transparent in the ultraviolet region of interest. Fortunately, optical transparency is not a limiting factor in the visible region around 409 nm. Thus the effects of a fairly large number of denaturants could be studied and compared by following the changes in absorbance of the heme moiety at the 409-nm maximum (Fig. 2). Where ORD measurements were possible within the experimental uncertainties of the technique, these measurements gave essentially the same denaturation midpoints as the absorbance measurements (Fig. 1).

Table 1 presents data on the myoglobin-denaturing power of 31 related amides, ureas, alcohols, and glycols. The reported solvent denaturation midpoint $S_{1/2}$, defined as the molar concentration of denaturant required to produce a 50-percent change in the particular experimental feature chosen to follow the denaturation transition (in our case, the absorbance change at 409 nm), is a relative measure of the effectiveness or denaturing power of the reagent used (*2, 5, 6, 19*).

In a particular series of related compounds, the denaturing power increases with increasing chain length or hydrocarbon content. As a rule, branching of the hydrocarbon portion of the denaturants tends to reduce activity or effectiveness. Thus the branched-chain alcohols appear to be less effective than the straight-chain alcohols of the same carbon content, and *N,N*-diisopropylacetamide is less effective than the straight-chain compound *N,N*-di-*n*-propylacetamide (Table 1). The chain length and degree of branching in alcohols are similarly related to their effects on the thermal stability of ribonuclease (*20, 21*) and on lysozyme (*22*). If the heteroatom (nitrogen in the case of the amides and ureas) is incorporated into branched chains or is otherwise crowded, it loses potency. In addition, for a given set of isomers of the same class (that is, the C_4 and C_5 amides or the C_5 ureas), compounds with shorter hydrocarbon branches or with a greater number of branches tend to be less effective denaturing agents. In contrast with the effect of increasing hydrocarbon content, increasing hydroxyl content tends to reduce the denaturing power of the solvent. Thus ethylene glycol is less effective than ethanol, and propylene glycol is substantially less

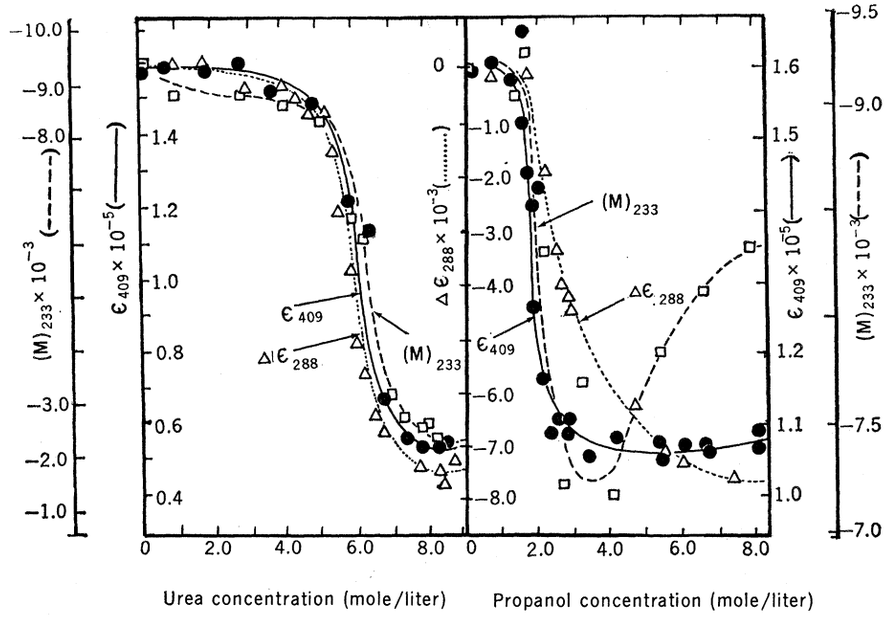

Fig. 1. Changes in optical rotation ($[M]_{233}$ at 233 nm, molar absorbance (ϵ_{409}) at the Soret band (409 nm) (the heme absorption band at 406 nm), and absorbance difference ($\Delta\epsilon_{288}$) at 288 nm accompanying denaturation by urea and propyl alcohol of sperm-whale myoglobin in 0.1*M* acetate (*p*H 5.7) at 25° ± 1°C.

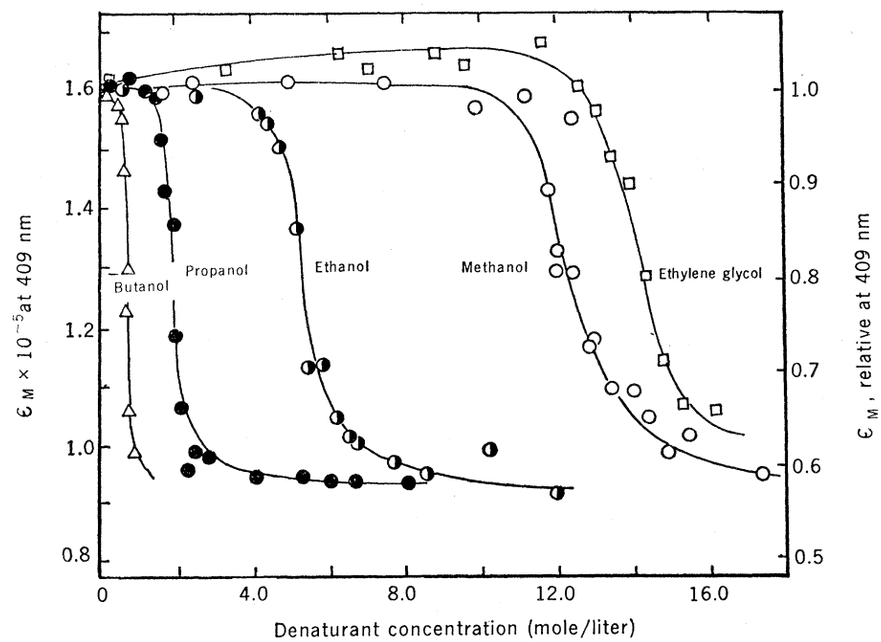

Fig. 2. The solvent denaturation of sperm-whale myoglobin by the straight-chain alcohols and ethylene glycol in 0.1*M* acetate (*p*H 5.7) at 25° ± 1°C.

effective than propanol. The increase in molecular weight due to the additional oxygen would not add to the molar volume of the denaturant sufficiently to account for the approximately three- to fourfold decrease in denaturing power on a mole-for-mole basis.

These findings indicate the importance of hydrophobic interactions, and in many ways they parallel the effects of denaturants on the stability of native DNA (*4–6, 19, 23*). Significantly, they also agree with what is known of the spatial distribution and location of the amino acid side chains in sperm-whale myoglobin. The x-ray crystallographic structure of this protein indicates that nearly all the hydrophobic side chains form the interior parts of the protein, whereas the surface of the protein contains mainly the remaining polar and ionizable groups (*24*). Considerations based on the free energies of transfer of hydrophobic side chains and peptide groups, from a solvent environment similar to the protein interior to another solvent environment (*25–28*), would provide argument that the less polar compounds with increasing hydrocarbon content should be more effective denaturants than the more polar ureas or glycols. The fact that the free energies of transfer of hydrophobic side chains and peptides from water to alcohol or water to urea solutions (*25*) are more negative than the transfer to ethylene glycol solutions (*26*) of the same molar concentration favors the more potent character of urea and ethanol.

Table 1. Correlation between the denaturing power and hydrocarbon content of various myoglobin denaturants in 0.1*M* acetate (*p*H 5.7) at 25° ± 1°C. The effectiveness or denaturing power of the reagent is given in terms of the denaturation midpoint.

Compound	Carbon series	Denaturation midpoint	
		Moles per liter	Percent (by volume)
Alcohols and glycols			
Methanol	C_1	12.4	50
Ethylene glycol	C_2	14.2	79
Ethanol	C_2	5.3	31
Propylene glycol	C_3	8.4	61
2-Propanol	C_3	3.4	26
1-Propanol	C_3	2.0	15
tert-Butanol	C_4	2.4	23
2-Butanol	C_4	.15	1.4
1-Butanol	C_4	.80	7.3
Ureas			
Urea	C_1	6.6	30
Methylurea	C_2	5.6	34
1,3-Dimethylurea	C_3	5.4	42
Ethylurea	C_3	3.8	28
Propylurea	C_4	1.8	13
Tetramethylurea	C_5	3.6	44
1,3-Diethylurea	C_5	1.9	21
Amides			
Formamide	C_1	9.4	37
Acetamide	C_2	7.7	39
N-Methylformamide	C_2	7.0	41
N,N-Dimethylformamide	C_3	7.8	60
Propionamide	C_3	4.4	31
N,N-Dimethylacetamide	C_4	7.5	71
N-Methylpropionamide	C_4	5.1	48
N-Ethylacetamide	C_4	3.0	28
Butyramide	C_4	2.3	19
N,N-Dimethylpropionamide	C_5	4.5	46
N,N-Diethylformamide	C_5	1.8	20
N-Butylacetamide	C_6	1.0	13
N,N-Diethylpropionamide	C_7	1.0	15
N,N-Diisopropylacetamide	C_8	.6	10
N,N-Di-*n*-propylacetamide	C_8	.3	5

The effects of blocking alkyl groups on the amino groups in ureas and amides are significant in relation to the relative importance of hydrogen bonding. At first glance, data on the ureas and amides obtained on a mole-for-mole basis indicate that alkyl substitution of these two classes of denaturants would also increase their denaturing ability. This is certainly true of the higher monoalkyl- and dialkyl-substituted amides and ureas: *N*-ethyl-, *N*-propyl-, and *N,N*-diethylurea, and the *N*-mono-, *N,N*-diethyl-, *N*-propyl-, and *N*-butylamides as measured on a mole-for-mole basis or in percent (by volume). Unfortunately, in the case of all compounds with methyl substituents at the nitrogen atoms (mono-, di-, and tetramethylurea and all the *N*-methyl and *N,N*-dimethyl–substituted amides), an ambiguity arises whether one expresses the denaturing power per mole or on a percentage (by volume) basis (or by use of any other meaningful scale) in the proper assessment of the relative effectiveness of the denaturant. The molar basis would be a more appropriate measure of direct interactions, such as the formation of hydrogen bonds or the exchange of the functional amide or carbonyl groups of the denaturant with similar groups of the protein polypeptide chain and amino acid side chains, whereas measurement in percentage (by volume) would perhaps more closely reflect the hydrophobic character of the denaturant and its effect on the structure of the solvent and the ensuing solubilization of hydrophobic side chains.

Perhaps the most striking feature of alkyl substitution is the apparently low activity of the methyl-substituted compounds on the volume-percent scale. The denaturation midpoint changes from 30 percent for urea to 42 and 44 percent for di- and tetramethylurea, respectively; correspondingly, the denaturation midpoint changes from 37 percent for formamide to 41 and 60 percent for the mono- and dimethyl-substituted derivatives. Methyl-substituted compounds exhibit low activity toward proteins (*20, 29*) and nucleic acids (*19, 23*). The tetramethylammonium ion has almost no effect on the thermal stability of DNA, or on the nuclear magnetic resonance frequency of water, as compared to other chaotropic (chaos-promoting) salts (*19*). Similarly, the tetramethylammonium ion does not essentially alter the thermal stability of ribonuclease, collagen, and myosin (*20*); however, in the case of ribonuclease (*20*), the higher ethyl-, propyl-, and butyltetraalkyl salts exhibited increased effectiveness, in agreement with the proportionally increasing importance of the hydrocarbon portion of the denaturant. In light of these findings, it would appear that the tendency for alkyl groups to promote structure formation of water (*30*) associated with hydrophobic interactions (*1, 9, 25, 26*) is partly canceled by the hydrogen-bonding potential of carbonyl and amide groups of the ureas and amides. Such effects should most strongly manifest themselves in the case of the lowest methyl-substituted series of compounds.

The effects of methyl substitution or replacement of the hydrogen atoms in amides and ureas may be interpreted as blocking or weakening the potential hydrogen-bond breaking sites with a concomitant loss of hydrophobic character, owing to the shortening and crowding of the hydrocarbon portion of the denaturant. This interpretation of the mode of action of ureas and amides is suggested by the comparison of the relative effectiveness of the methyl-substituted and straight-chain C_3 and C_4 amides of the same formula weight (that is, the denaturing power of *N,N*-dimethylformamide as compared to propionamide, and that of *N,N*-dimethylacetamide as compared to *N*-methylpropionamide and butyramide).

THEODORE T. HERSKOVITS
HELENE JAILLET
Department of Chemistry, Fordham University, New York 10458

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).

31. Supported by research grant GM-14468 from NIH.

11 October 1968

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

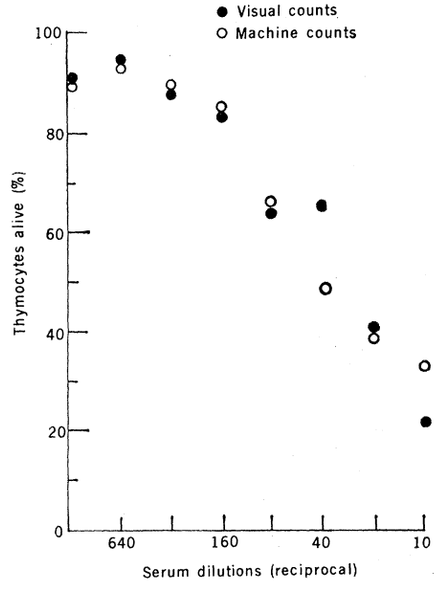

Fig. 1. Cytotoxic test on mouse thymocytes with H-2 isoantiserum showing close similarity of visual and automatic counts. Reagents were deliberately not filtered on this occasion in order to show the error due to particulate matter that scatters sufficient light to generate signals adding to the live 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.