tern of temperature dependence largely similar to that for maximum output (Fig. 1B).

Changes in temperature between 30° and 40°C have little effect on normal CM (*9, 10*), in agreement with results presented here. However, Bornschein and Krejci (*11*) found that the rate of loss of CM in anoxia is decreased at lower temperatures. Fernández *et al.* (*9*) suggested that during oxygen deprivation, hypothermia reduces the metabolic rate of the generators of CM, the hair cells and vascular stria (*7*), and thereby prolongs survival of the response, presumably by sparing energy reserves (*12*). The noise-induced loss of CM (Fig. 1) exhibits temperature dependence in the same direction as does CM loss in anoxia, but over a much more extended time period (*11*). While anoxia affects both hair-cell and strial generators (*13*), noise affects mainly the hair cells and influences the stria to a lesser extent (*2*).

The CM is thought to arise as ion current by modulation of a voltage gradient across the hair cells in the reticular lamina (*7*). It is therefore likely that continuous acoustic stimulation results in redistribution of ions across hair-cell membranes. Undoubtedly, energy is required for ion transport to maintain an electrical gradient between the interior of the hair cells and the external fluid space of scala media (*7*). The decreased rate of noise-induced reduction of CM at lower temperatures and the increased rate at higher temperatures may result from differential use of energy stores by active transport and temperature-dependent metabolic processes in the cochlea. Structural changes that do not affect normal response may also be involved. In these experiments, interpretations employing temperature coefficients or Arrhenius energies of activation are made difficult by the invariance of CM with temperature before exposure to noise, the initial retardation of noise-induced loss of CM at 29°C, and the greater change with temperature in rate of loss above 37°C than below 37°C. However, the mechanism of noise-induced reduction of cochlear response apparently involves processes markedly dependent on body temperature.

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## Gelation of Sickle Cell Hemoglobin: Effects of Hybrid Tetramer Formation in Hemoglobin Mixtures

Abstract. *The altered gelation behavior found in mixtures of sickle cell hemoglobin with other hemoglobins is due to the formation of hybrid hemoglobin tetramers from unlike dimers. The hemoglobins need not possess the deoxy quaternary structure for gelation to occur; liganded forms are also capable of participation in gelation.*

If erythrocytes from patients with sickle cell anemia are deoxygenated, aggregation of their sickle cell hemoglobin, HbS (*1*) occurs, which produces the distortion of the erythrocyte into the characteristic sickle or holly-leaf shape. If the hemoglobin from these patients is purified and deoxygenated, similar aggregation may occur, which leads to the formation of a solid phase, a nematic gel. Gelation is highly dependent on hemoglobin concentration; the critical concentration, below which no gelation will occur, is known as the minimum gelling concentration (MGC) and is a function of other variables such as temperature, *p*H, ionic strength, and presence of organic phosphates. In addition, determination of the structural features which the hemoglobin must possess if this gelation is to occur is central to an understanding of the molecular mechanism of sickling.

A productive approach to this problem has been adopted by Bookchin, Nagel, and Ranney (*2*), after the initial studies of Singer and Allison (*3*). They have studied the dependence of the MGC on the composition of a series of deoxygenated mixtures of HbS with other, non-S hemoglobins (HbA, HbF, HbC Harlem, and Hb Korle-Bu) and with CNmet-HbS. In this way, the extent of interaction between these other hemoglobins, with different structures, and normal deoxy-HbS can be readily determined. In short, they find that in mixtures of these hemoglobins with HbS, the MGC increases, but the partial concentration of HbS at gelation decreases. These other hemoglobins are therefore able to replace HbS in gelation, in a manner that is still unknown.

A recent quantitative analysis of such data (*2*) by Minton (*4*) assumes that aggregation proceeds via two steps: the first involves linear polymerization of hemoglobin tetramers into filaments, and the second, side-by-side aggregation of these filaments to form the gel. These two steps are assumed to be affected differently by the nature of the amino acid side chains at positions 6$\beta$ (Glu in HbA and Hb Korle-Bu; Val in HbS and HbC Harlem), and at 73$\beta$ (Asp in HbA and HbS; Asn in HbC Harlem and Hb Korle-Bu), and by the quaternary structure of the hemoglobins. I demonstrate here that these data (*2*) may be fitted quantitatively by a somewhat simpler model, based on the presence of hybrid hemoglobin tetramers formed by association of unlike dimers in such binary mixtures (*5*). Further, I show experimentally that if the formation of such hybrid tetramers is prevented, then the MGC is increased. The role of hybrid tetramer formation in the gelation of binary hemoglobin mixtures has been discussed (*2, 6, 7*), but no quantitative analysis or experimental test has hitherto been applied.

The following dimer-tetramer association equilibria occur in a binary mixture of hemoglobins (for example, that formed by HbA and HbS):

$$T_A \rightleftharpoons 2\,D_A;\ K_A = [D_A]^2/[T_A]$$
Dissociation of HbA

$$T_S \rightleftharpoons 2\,D_S;\ K_S = [D_S]^2/[T_S] \quad (1)$$
Dissociation of HbS

$$T_{AS} \rightleftharpoons D_A + D_S;\ K_{AS} = 2[D_A][D_S]/[T_{AS}]$$
Dissociation of HbAS hybrid

where T and D denote hemoglobin tetramers and dimers, respectively. If the total concentrations of HbA and HbS, $[H_A]$ and $[H_S]$, are known, then the concentrations of $T_A$, $T_S$, $T_{AS}$, $D_A$, and $D_S$ may be derived in terms of these two quantities and the three dissociation constants $K_A$, $K_S$, and $K_{AS}$ (*8*). I now assume that all three tetrameric species are, in principle, capable of incorporation into the gel, and that gelation will occur when the weighted sum of their concentrations reaches a critical value $C$, the concentration at which pure deoxy-HbS gels under the same experimental conditions. That is,

$$w_1[T_S] + w_2[T_{AS}] + w_3[T_A] = C \quad (2)$$

where the $w_i$ are the weights; $w_1$, the weight assigned to the deoxy-HbS tetramer, is set equal to 1.00 throughout. These weights resemble relative association constants for addition of the tetramers to the gel; the higher the weight, the more readily that tetramer is incorporated (*9*).

In all previous experiments (*2*), the hemoglobins were mixed in the oxy form, in which attainment of the new tetramer-dimer equilibrium and formation of hybrid tetramers is rapid, and the mixture was then deoxygenated. The resultant deoxy-Hb tetramers have extremely small dissociation rate constants, and hence any further redistribution of the homogeneous and hybrid tetramers can take place only very slowly (*10*). The distribution established on mixing in the oxy form is thus effectively frozen in by subsequent deoxygenation.

In order to analyze the data further, values of $K_A$, $K_S$, and $K_{AS}$ for the oxy forms were required. Sedimentation equilibrium ultracentrifugation was carried out on the liganded forms of HbA, HbS, and an equimolar mixture of HbA and HbS, in which hybrid formation would be maximized. All three samples were found to have identical dissociation constants of $2.0 \pm 0.5\ \mu M$; thus, $K_A = K_S = K_{AS} = K$. From this single value, the values of $[T_A]$, $[T_S]$, and $[T_{AS}]$ were calculated (*8*) for each data point (*2*). These values were substituted in Eq. 2, and the weights $w_2$ and $w_3$ were determined by a least-squares procedure. The weights thus derived for each tetramer are shown in Table 1, and the agreement between the curves calculated from these weights and their experiments is illustrated in Fig. 1, A to D.

It is likely that a variety of reasonable two-parameter theories would be capable of fitting experimental data of the simple form shown. Confirmation of the central role of hybrid tetramers was therefore sought by repeating certain of the experiments of Bookchin and Nagel (*2*), with the important modification that the hemoglobins were mixed in the deoxy form, rather than the oxy. The low rate of dissociation of deoxy-Hb tetramers ensures that essentially no hybrid tetramers are present (*10, 11*). From the weights for the homogeneous deoxy-HbA and deoxy-HbS tetramers previously derived, it was predicted that mixing in the deoxy form, with no hybrids present (Fig. 1E, upper curve) would result

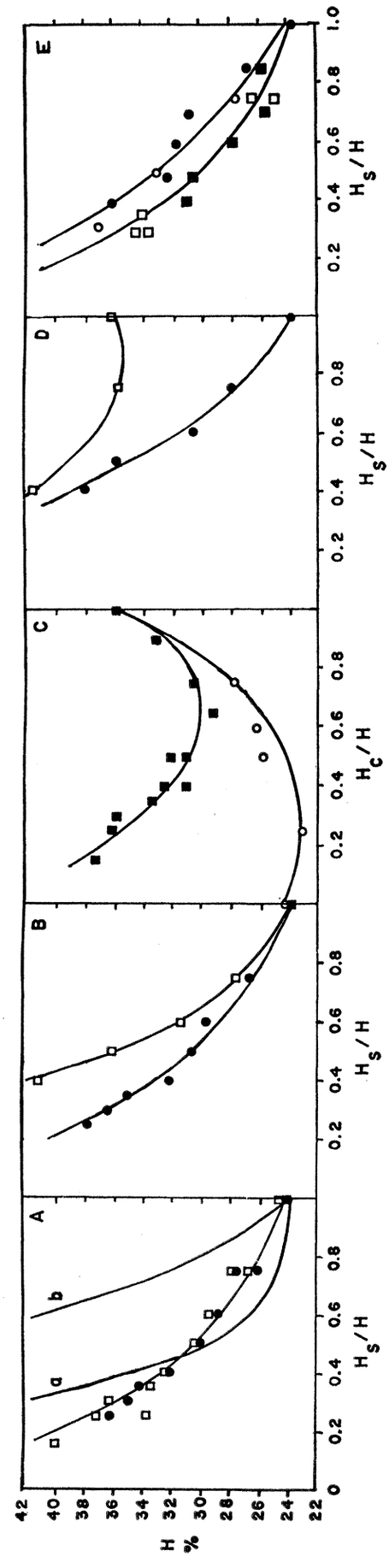


Fig. 1. The dependence of the minimum gelling concentration, denoted H, on the fractional composition of deoxygenated mixtures of hemoglobins. All data points in (A) to (D) are from (*2*); those in (E) are from this work (*12*). The solid lines (A to D) are theoretical curves, given by Eq. 2, with the appropriate weights taken from Table 1. (A) □, Deoxygenated mixtures of oxy-HbS with oxy-HbA; ●, CNmet-HbA. Curve a is the best fit which can be achieved if it is assumed that homogenous deoxy-HbA tetramers are completely excluded. Curve b is obtained if it is assumed that there is no interaction whatever between HbS and the other hemoglobin. (B) □, Deoxygenated mixtures of oxy-HbS with oxy-HbF; ●, with CNmet-HbF. (C) ○, Deoxygenated mixtures of oxy-HbC Harlem with oxy-HbS; ■, with oxy-HbA. (D) ●, Deoxygenated mixtures of oxy-Hb Korle-Bu with oxy-HbS; □, with oxy-HbC Harlem. (E) ○ and ●, HbA and HbS mixed in the deoxy form; □ and ■, HbA and HbS mixed in the oxy form and subsequently deoxygenated. Points denoted ● and ■ were obtained on the same sample (*12*). The solid lines are theoretical curves; the lower is that derived from the corresponding Bookchin and Nagel data (A), and the upper is that derived on the assumption that no hybrid species are present [see text and (*12*)]. Although the conditions used in these experiments differ slightly in *p*H, molarity, and nature of buffer from those in (*2*), the results are in agreement.

in a higher MGC than mixing in the oxy form, with hybrids present (Fig. 1E, lower curve). This was confirmed by experiment (Fig. 1E) (*12*). Minton's model (*4*) does not readily account for this result.

The structural implications of the weights, or relative association constants, derived for each tetramer (Table 1) are complex, and no clear picture emerges. The weights for the A-S and F-S hybrids do not depend on the state of ligation of the non-S chains, and do not differ greatly from 1.00. This suggests that these hybrids are capable of adopting the normal deoxy-Hb quaternary structure, and that only one of the $\beta$ chains need be derived from HbS for gelation to occur (*2*). Considering only the deoxy forms, close examination of the weights derived for HbS, HbA, HbC Harlem, Hb Korle-Bu, and all possible hybrids formed between them reveals no underlying order, such as might be expected if, for example, the residues at $6\beta$ and $73\beta$ were involved in two different types of interaction, in two different interfaces, on gelation. Residues other than these must also be involved. Curiously, possession of only one $73\beta$ Asn per tetramer (as in hybrids involving deoxy-HbC Harlem and deoxy-Hb Korle-Bu) facilitates gelation, but possession of two (as in homogeneous deoxy-HbC Harlem and deoxy-Hb Korle-Bu tetramers) hinders it.

Since hemoglobin contains a twofold axis of symmetry, hybrid formation results in a loss of molecular symmetry, as the two halves of the molecule are no longer equivalent. Nevertheless, the weights of the the hybrids are not reduced to 0.5, which would be expected if only half of each hybrid molecule could interact. The half containing the non-S chains must also be capable of interaction.

This point is further demonstrated by the incorporation of the homogeneous deoxy-HbA, CNmet-HbA, and CNmet-HbF tetramers. The weights derived for deoxy-HbA and CNmet-HbA are almost identical, despite their very different quaternary structures; in contrast, CNmet-HbF is equivalent to CNmet-HbA, but deoxy-HbF is completely excluded (*13*). The $\gamma$ chain of HbF differs in 39 locations from the $\beta$ chain in HbA and HbS, and many of these differences are clustered round residues $6\beta$ and $73\beta$. The exclusion of deoxy-HbF may be due to the particularly unfavorable nature of one of these differences; for example, residue 76 is Ala in the $\beta$ chains, and Lys in the $\gamma$ chains. The incorporation of both CNmet-HbF and CNmet-HbA, albeit with low weights, was unexpected; it has generally been believed that the deoxy quaternary structure is required for gelation or sickling. However, attempts to fit the data by setting the weights of CNmet-HbF and CNmet-HbA to zero (no incorporation) resulted in systematic misfits between theory and experiment (Fig. 1A, curve a). This finding raises the possibility that other liganded forms may be incorporated, such as oxy-HbS. If this is indeed the case (as is suggested by preliminary experiments), then the hitherto puzzling observation that erythrocytes may sickle even at high levels of saturation with oxygen (*14*) may be explained.

Table 1. Relative association constants. These weights were derived as described in the text and in (*8*) from the data in (*2*); all experiments were carried out in 0.15*M* potassium phosphate, *p*H 7.35, at 25°C. Standard errors derived from the least-squares procedures are shown. The possibility of systematic errors due to deviations from ideality is noted in (*9*).

| Species | Weight |
|---|---|
| *Homogeneous tetramers* | |
| deoxy-HbS | 1.00 |
| deoxy-HbA | 0.50 ± 0.18 |
| CNmet-HbA | 0.53 ± 0.19 |
| deoxy-HbF | 0.11 ± 0.16 |
| CNmet-HbF | 0.47 ± 0.18 |
| deoxy-HbC Harlem | 0.67 ± 0.20 |
| deoxy-Hb Korle-Bu | 0.30 ± 0.20 |
| *Hybrid tetramers* | |
| deoxy-HbS and deoxy-HbA | 0.80 ± 0.07 |
| deoxy-HbS and CNmet-HbA | 0.81 ± 0.06 |
| deoxy-HbS and deoxy-HbF | 0.79 ± 0.05 |
| deoxy-HbS and CNmet-HbF | 0.81 ± 0.07 |
| deoxy-HbS and deoxy-HbC Harlem | 1.09 ± 0.10 |
| deoxy-HbA and deoxy-HbC Harlem | 0.95 ± 0.07 |
| deoxy-HbS and deoxy-Hb Korle-Bu | 0.73 ± 0.07 |
| deoxy-HbC Harlem and deoxy-Hb Korle-Bu | 0.75 ± 0.15 |

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### References and Notes

1. Abbreviations used: Hb, hemoglobin; deoxy-Hb, deoxyhemoglobin; oxy-Hb, oxyhemoglobin; CNmet-Hb, cyanide methemoglobin; Ala, alanine; Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Lys, lysine; Val, valine.
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8. Set $K_A = K_S = K_{AS} = K$ (see text). Then it may be shown that

$$[T_A] = [D_A]^2/K;\ [T_{AS}] = \tfrac{1}{2}[H_A] - [D_A] - 2[D_A]^2/K:$$
$$[D_S] = \{K/(2[D_A])\}\ (\tfrac{1}{2}[H_A] - [D_A] - 2[D_A]^2/K)$$
$$[T_S] = \{K/(4[D_A]^2)\}\ (\tfrac{1}{2}[H_A] - [D_A] - 2[D_A]^2/K)^2$$
$$[H_S] = \{[H_A]^2K/(4[D_A]^2)\} - \{[H_A]K/(2[D_A])\} - [H_A]$$

The last equation is solved for $[D_A]$ in terms of the given values of $K$, $[H_A]$, and $[H_S]$; $[D_A]$ is then inserted in the other equations.
9. This approach implicitly assumes that gelation is a highly cooperative process, for which there is evidence [R. C. Williams, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1506 (1973)]. It is to be contrasted with the assumption in Minton's treatment (*4*), that gelation occurs when the concentration of a single *n*-fold polymer of hemoglobin tetramers reaches a critical value. It should also be stressed that neither Minton's nor my analysis considers deviations from ideality, and these are considerable in these highly concentrated protein solutions. Such deviations appear to arise principally from excluded-volume effects (R. C. Williams, Jr., *ibid.*); hence, they depend mainly on the total hemoglobin concentration and only to a lesser extent on the distribution of tetrameric species. The distribution of concentrations calculated from the equations in (*8*) is therefore probably valid, but the absolute values may be in error. Until the effects of non-ideality are quantified, any interpretation based on the absolute values of these weights must be tentative.
10. The dissociation equilibrium constant of deoxy-HbA at *p*H 7.0 has been estimated indirectly as $3 \times 10^{-12}M$ [J. O. Thomas and S. J. Edelstein, *J. Biol. Chem.* **247**, 7870 (1973)]. The association rate constant is $6.3 \times 10^5 M^{-1}\,\text{sec}^{-1}$ [M. E. Anderson, J. K. Moffat, Q. H. Gibson, *ibid.* **246**, 2796 (1971)]. Hence, the estimated rate constant for dissociation of the deoxy-Hb tetramer is $2 \times 10^{-6}\,\text{sec}^{-1}$; the half-life for dissociation is about 40 hours. This is probably an upper estimate, but the half-life is certainly long with respect to the time taken to determine the MGC.
11. The initial concentration of hybrid tetramers after mixing is negligible, as the concentrations of deoxy-Hb dimers before mixing is so small. After being mixed, hybrid deoxy-Hb tetramers will appear at a rate given by the slower of the dissociation rates of the deoxy-HbA and deoxy-HbS tetramers. Since the generation of these hybrids affects the MGC, the rate of dissociation may in principle be derived from the time dependence of the MGC.
12. Both HbA and HbS were prepared according to the method of Q. H. Gibson [*J. Biol. Chem.* **245**, 3285 (1970)]; the HbS was obtained by Dr. S. Charache from patients homozygous for sickle cell hemoglobin, and was adjudged by eletrophoresis to contain less than 5 percent HbF. Both hemoglobins were dialyzed against 0.05*M* ammonium phosphate, *p*H 7.0, and concentrated by pressure dialysis in an Amicon cell. Deoxygenation was accomplished by placing the samples in a rotating flask which was continually flushed with purified nitrogen. For determination of the MGC, appropriate volumes of the deoxy samples were mixed in a 50-ml round-bottom flask, with oxygen being completely excluded. The concentration was increased until gelation occurred by rotating the flask in a controlled temperature bath and flushing with dry nitrogen. The flask was cooled to liquefy the gel, exposed to air, and a sample was removed for measurement of the hemoglobin concentration. A small amount of air-equilibrated buffer was added to dilute and completely oxygenate the sample, which was then deoxygenated and concentrated once more until gelation occurred. The final concentration

was measured as before. In this way, an accurate measure of the difference in MGC as the same composition of the mixture could be obtained. Although this procedure involved gelling the sample twice, no hysteresis was detected; data obtained on once-gelled samples (denoted by open symbols in Fig. 1E) or twice-gelled samples (closed symbols in Fig. 1E) are in good agreement.

13. This result as first sight conflicts with the conclusions of Bertles *et al.* (*6*), who found that deoxygenation of HbS-HbF mixtures apparently led to the complete exclusion of HbF from the solid phase. However, it is likely that under their conditions gelation occurred before complete deoxygenation, and that the HbS was preferentially deoxygenated. Patients homozygous for HbS and with hereditary persistence of HbF have a hemoglobin composition of 70 percent HbS and 30 percent HbF; those who are heterozygous for HbS and with β-thalassemia have a composition of 70 percent HbS, 30 percent HbA. The latter exhibit the symptoms of sickle cell anemia, but the former do not. This result is now explicable: deoxy-HbA tetramers may be incorporated into the gel, but deoxy-HbF tetramers may not.
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15. Supported by NSF grant GB 32231X. I thank Dr. S. Charache for the sickle-cell blood, Preston Hensley for performing the ultracentrifuge experiment, and Drs. Q. Gibson, S. Edelstein, R. Nagel, and S. Charache for discussions.

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## Extracellular Acidosis Protects Ehrlich Ascites Tumor Cells and Rat Renal Cortex against Anoxic Injury

Abstract. *The present study indicates that extracellular acidosis protects Ehrlich ascites tumor cells and rat kidney cortex cells against injury from anoxia. Parameters measured included cell potassium, adenosine 5′-triphosphate, and uptake of vital dyes. Cells survived longer at a* p*H of 5.6 to 6.5 than at a* p*H of 7.4;* p*H 7.9 was most detrimental. These findings indicate that production of protons by anoxic cells may be a protective feedback mechanism.*

The tolerance of cells to injury from anoxia varies greatly in different cell types. Morphological and biochemical effects of anoxia have been widely studied both in vivo and in vitro, but methods to protect the function or prevent injury or death of the cells due to anoxia or to other lethal agents are still quite limited and little studied. It is generally accepted that extracellular acidosis is detrimental to the function of many organs. However, little attention has been paid to the effect of acidosis on the viability or death of cells in an anoxic or an aerobic milieu. Recently it has been found that the recovery of heart muscle function following hypoxia was protected by acidosis (*1*). The present experiments were designed to determine the effects of anoxia and acidosis on the viability of Ehrlich ascites tumor cells (EATC) and rat kidney cortical cells in vitro. Vital dye uptake, intracellular potassium, and adenosine 5′-triphosphate (ATP), known to be good indicators of cell injury or cell death caused by several mechanisms (*2*), were used as parameters for EATC, and potassium was used for kidney cells. Ehrlich ascites tumor cells were cultivated in the peritoneal cavity of the mouse by weekly transplantation and handled as reported earlier (*3*). The cells were collected 7 or 8 days after inoculation, washed two times in a modified Krebs-Ringer phosphate (KRP) buffer (*4*), centrifuged at 71*g* for 5 minutes, and resuspended in KRP so that the final concentration was $1.0 \times 10^7$ to $1.6 \times 10^7$ cells per milliliter. Cells preincubated aerobically for 10 minutes at 37°C were used as preanoxia controls. Renal cortical slices were prepared with a Stadie-Riggs microtome. Slices were cut within 1 minute after removing the kidney and immediately placed into the incubation chamber in KRP or were used as preanoxia controls. Slices were about 0.2 mm thick, as calculated by determining their areas and weights and assuming that the tissue density was 1 (*5*). An airtight chamber, maintained at 37°C, containing 15-ml plastic vessels filled with appropriate incubation media was used for anoxia experiments; a water bath with 45 oscillations per minute was also used. Relative humidity inside the chamber was maintained at 100 percent. The chamber and the incubation media were gassed before being used, and the chamber was gassed during experiments with high-purity grade dry nitrogen in order to totally remove oxygen. Oxygen content was measured before and during experiments with an oxygen monitor (model 53, Yellow Springs Instrument). The trace amount of residual oxygen was consumed during the first 2 minutes of incubation when EATC, preincubated under aerobic conditions and suspended in the buffer vehicle, were injected into an anoxic milieu. Portions were taken from the samples at intervals up to 12 hours. The EATC viability was determined in a hemacytometer chamber with nigrosin as the vital dye stain (*6*); ATP was analyzed in perchloric acid–

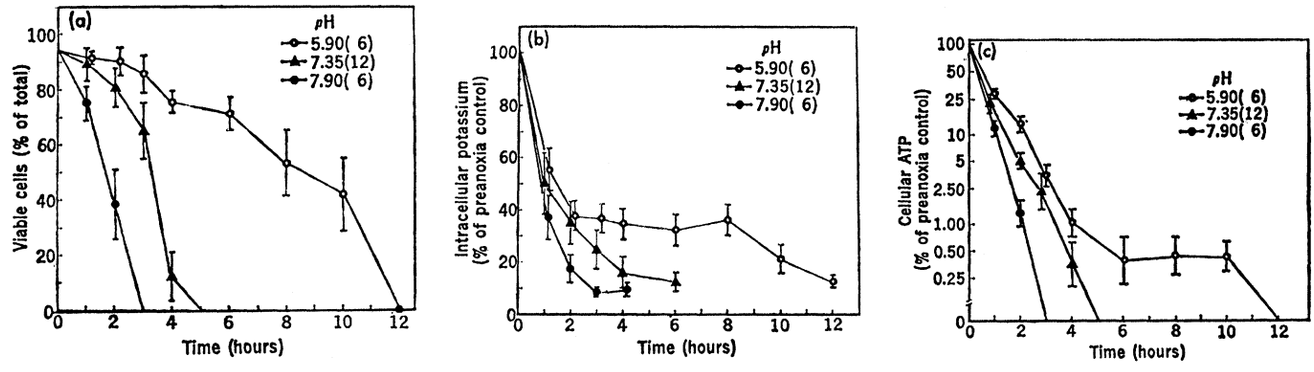


Fig. 1. (a) Viability of Ehrlich ascites tumor cells (EATC) during 12 hours of anoxia at varying *p*H values. The mean preanoxia count of nigrosin-stained cells was 6.6 ± 2.7 percent of the population. The numbers in parentheses following the *p*H values indicate the number of the experiments in each group. Brackets indicate 1 standard deviation. (b) Intracellular potassium of EATC during 12 hours of anoxia. The samples were taken from the same incubations as in (a). The mean preanoxia content of intracellular potassium was 1.84 ± 0.38 μmole per $10^7$ cells (100 percent). (c) Intracellular adenosine 5′-triphosphate (ATP) of EATC during 12 hours of anoxia. The mean preanoxia content of cellular ATP was 5.39 ± 8.2 μmole per $10^7$ cells.