Nonelectrolyte Transport in Muscle during Induced Protein Loss

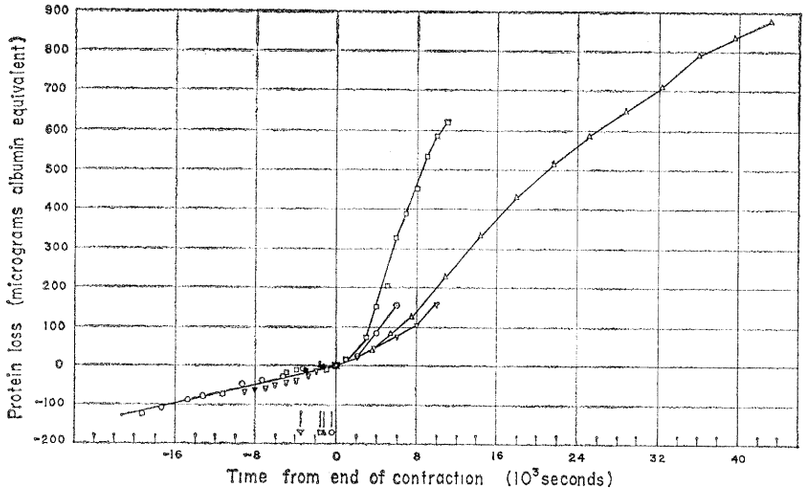
Abstract. *The accelerated loss of protein from* Rana pipiens *sartorius muscle following the induction of rigor by 100 millimolar 1-pentanol is not accompanied by increase in the rate constant for the efflux of urea. This observation is not readily accounted for by classical lipid-pore membrane permeability models. Alternative models are considered, in which protein and nonelectrolyte transport are dissociated; the dissociation could be (i) topological, due to compartmentalization, or (ii) functional, for example, if protein loss were due to change of the state of intracellular protein or if urea transport were limited by diffusion in the cytoplasm.*

During a study of the influence of relatively nonpolar molecules on transport in skeletal muscle, we have made certain observations which appear inconsistent with, and thus cast doubt on, classical membrane models of permeability. In these models (*1, 2*) it is maintained that cells are surrounded by a thin cortical structure, the plasma membrane, whose properties determine the rates at which molecules enter and leave the cell. This membrane is lipoprotein in composition and contains small aqueous channels (pores). Flux rates of nonelectrolytes are determined by the rate of permeation through the membrane. Nonpolar and weakly polar solutes are able to dissolve in the membrane substance and therefore permeate relatively rapidly. Highly polar solutes are poorly soluble in the membrane and therefore permeate slowly, unless, as in the case of water, they are sufficiently small to pass through the pores.

In these models, soluble intracellular protein molecules, which are both large and highly polar, are confined within the cell by the membrane. When protein is lost, as by "normal" leakage (*3, 4*) or during lysis (*1*, ch. 18; *5*) by any of various agents (for example, osmotic shock, metabolic poisons, narcotics, detergents, specific lysins, mechanical agitation), the loss is attributed to the presence or formation of enlarged pores or to the rupture of the membrane. Since the same membrane structure is assumed to both limit protein loss and determine the flux rates of small nonelectrolytes, these transport phenonema should be correlated: a significant increase in the rate of protein loss from the cell would seem to imply a concomitant increase in the flux rate of smaller polar molecules (*4*). In this report we describe a system in which protein loss and nonelectrolyte transport are dissociated.

The sartorius muscle of *Rana pipiens* was used. Efflux rate constants of nonelectrolytes (*6*) from this tissue show a pattern of specificity to which the membrane model just described is applicable: for example, 1-propanol and urea, compounds of approximately equal molecular size, are weakly and highly polar, respectively; their rate constants at 15.2°C are 3.7×10^{-3} sec^{-1} and 3.4×10^{-4} sec^{-1}, which differ by a factor of 11. Water on the other hand, while highly polar, has a small molecular size and shows a rapid rate constant of 5.1×10^{-3} sec^{-1} at 5.6°C.

Protein loss from the sartorius muscle was induced by 1-pentanol, a cellular narcotic. The entry of this agent into the muscle is very rapid, more than 90 percent equilibration being reached in 500 seconds at 15.2°C (*6*). The equilibrium distribution of 1-pentanol between cellular and extracellular water is 0.98 for concentrations between 10 m*M* and 100 m*M*. This rapid and full entry precludes osmotic effects on the time scale appropriate to urea and protein fluxes (Figs. 1, 2, and 3). At low concentrations (less than about 60 m*M*) 1-pentanol, in a glucose-phosphate Ringer solution, produces fully reversible narcosis, as determined by the rheobase for electrical stimulation (*7*). Higher concentrations cause the muscle to go into rigor; in 100 m*M* 1-pentanol, at 15.2°C, rigor usually develops in 5 to 50 minutes. There is a small loss of protein from relaxed muscle, inde-



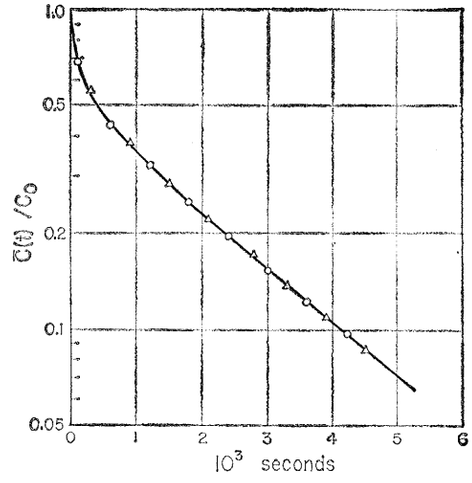


Fig. 1 (left). Loss of protein from *R. pipiens* sartorius muscle at 15.2°C before, during, and after development of rigor in 100 m*M* 1-pentanol (*8*). Both the ordinate and abscissa scales are taken relative to the time at which full rigor had developed. The time of introduction of 100 m*M* 1-pentanol is indicated by a solid symbol; the onset of rigor, by the short vertical lines along the abscissa. The units of protein loss are relative, since bovine serum albumin was used as the standard in protein estimation, and the Folin test may differ in its sensitivity to albumin and cellular proteins. Fig. 2 (right). Efflux of urea-C^{14} from *R. pipiens* sartorious muscle at 15.2°C (*2*). Open circles, efflux from relaxed muscle; open triangles, efflux from muscle equilibrated with tracer after full development of rigor. On the ordinate scale, $\bar{C}(t)/C_0$ is the mean tracer concentration divided by the tracer concentration in the tissue at time zero. To permit comparison of the form of the kinetics, efflux curves having the same flux-rate constant, S_a, were selected for presentation.

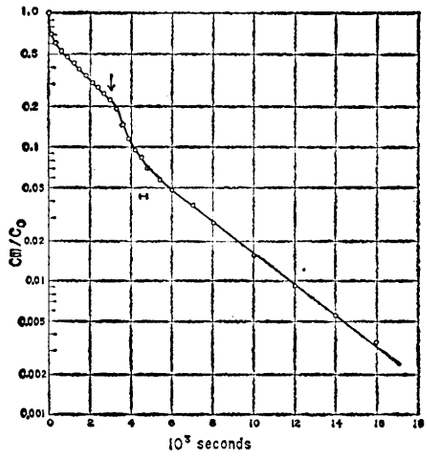


Fig. 3. Efflux of urea-C^{14} from *R. pipiens* sartorius muscle at 15.2°C before, during, and after development of rigor in 100 m*M* 1-pentanol. $\bar{C}(t)/C_0$ is the mean tracer concentration divided by the tracer concentration in the tissue at time zero. The time period indicated by the bar is that during which rigor is developing.

pendent of the concentration of 1-pentanol; however, following development of rigor there begins a more rapid efflux of protein which in time leads to a loss of about 20 percent (18.5 ± 2.3 percent in 24 hours in experiments with paired muscles) of the dry weight of the muscle. The nature of this loss may be seen by microscopic examination of muscle in rigor for short and long periods. Figure 4 shows that during the 24-hour period after development of rigor there is uniform diminution in the dry cross-sectional area of all muscle fibers, rather than a selective massive loss of material from a few fibers. Protein loss may therefore be treated as a property of the individual "typical" fiber rather than of a population. The time sequence of loss of protein from the sartorius is shown in Fig. 1 (*8*); each curve represents a muscle which was washed in successive portions of Ringer solution at 15.2°C until the time noted by a solid symbol, at which point the wash solutions were changed to Ringer containing 100 m*M* 1-pentanol. Accelerated protein loss began 1000 to 2000 seconds after rigor developed completely.

According to the classical membrane hypothesis, the period of protein loss is one in which the pore size is greatly increased, so that highly polar monomeric solutes which are too large for free passage through the pores in normal muscle should also show an increase in flux rate. Urea is a suitable solute to test this point, since it is highly polar, has a very slow flux rate in normal muscle (*9*), and has an equilibrium distribution virtually of unity between cellular and extracellular water in both relaxed muscle and muscle in rigor. A typical curve for the efflux of urea-C^{14} from the sartorius at 15.2°C is shown in Fig. 2 (open circles). Reversible narcosis caused by 1-pentanol increases the efflux rate of urea, as shown in Fig. 5, in which the ratio of the rate constant, S_e, in the presence of 1-pentanol to that in the absence of 1-pentanol (3.42×10^{-4} sec^{-1}) is plotted as a function of the 1-pentanol concentration.

Figure 3 shows the urea efflux in a typical experiment in which rigor and subsequent protein loss occur. The design of the experiment was the same as that for Fig. 1, in which the protein loss was followed. At the beginning of the experiment the muscle was washed in pentanol-free Ringer solution, the curve being comparable with that in Fig. 2. After the curve became rectilinear, the wash solution was changed to include 100 m*M* of 1-pentanol (arrow). The efflux rate immediately began to increase, reaching a maximum of 2.4 times the rate before the addition of 1-pentanol (mean of three experiments, range 2.2 to 2.9). As shown in Fig. 5, this represents the approximate value to which the reversible narcosis would extrapolate at a concentration of 100 m*M* 1-pentanol. At this point, development of rigor began, and was invariably associated with reduction in the efflux rate of urea, which then became constant for the duration of the experiment at a value of 0.87 times the original flux rate (mean of four experiments, range 0.69 to 1.1).

Comaprison of Fig. 3 with Fig. 1 permits some significant observations: The two periods of change in the efflux rate of urea do not correlate with the periods of change of protein loss; the initial change, an increase in S_e on addition of 1-pentanol, is associated with reversible narcosis; the final change, a decrease of S_e to a value smaller than that before addition of 1-pentanol, is associated with the change from the state of relaxation to that of rigor, and may be ascribable to the increasing muscle-fiber radius during development of rigor. Both of these changes in S_e occur before pro-

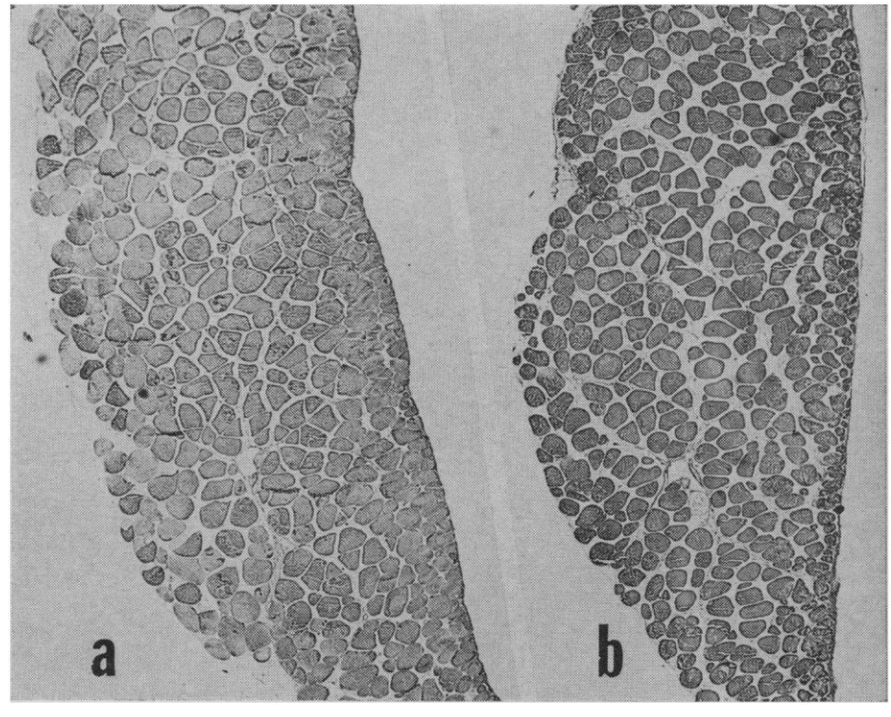


Fig. 4. Photomicrographs of *R. pipiens* sartorius muscle in which rigor was induced by 100 m*M* 1-pentanol. Paired muscles from a single frog were used. Muscle *a* was fixed in Susa at 15 minutes, and muscle *b* at 24 hours, after the development of full rigor. The tissues were desiccated in alcohol, embedded in paraffin, sectioned, and stained with eosin-hematoxylin (× 40). The individual fibers in *b* have shrunk relative to those in *a*, as indicated by their smaller size and more extensive extracellular spaces. There is no evidence of cell "ghosts" which would indicate the massive loss of protein from individual fibers.

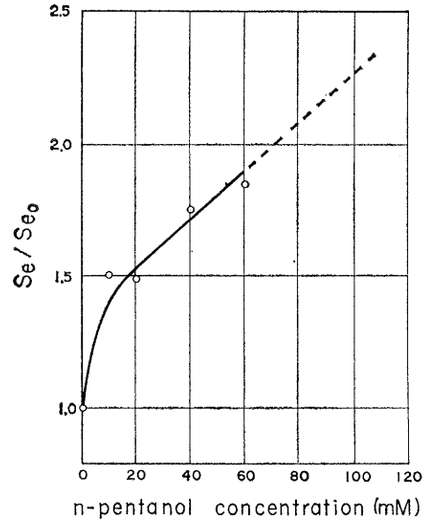


Fig. 5. The influence of 1-pentanol on the efflux of urea-C^{14} from the sartorius of *R. pipiens* at 15.2°C. The ordinate is the ratio of the efflux rate constant, S_e, in Ringer solution containing 1-pentanol to that, S_{e_0}, in the absence of 1-pentanol.

tein loss begins to accelerate. Further, and most important, during the period in which the rate of protein loss increases by a factor of at least 5, the efflux rate of urea remains constant and smaller than the control rate.

It appears from these results that increased loss of protein from muscle can occur without concomitant increase in flux of urea. This dissociation of protein and nonelectrolyte transport is not expected in classical membrane models, and it is not clear whether, by invoking heterogeneity of pore size and response to narcotics and rigor, the model of a single lipid-pore membrane can be reconciled with this dissociation. However, a number of other interpretations of our experiments are possible.

In one explanation, the dissociation of transport would be accounted for by assuming a topological compartmentalization of soluble protein in the cell. Different transport barriers would be encountered by protein and urea, and the dissociation of transport could be attributed to the differential influence of 1-pentanol on the various barriers. The details of such a model would depend on the specific topology assumed; for example, the soluble 20 percent of the cellular protein might be located entirely outside the principal barrier to urea transport, or, conversely, within a compartment entirely within the urea barrier. Electron-microscopic examination of the changes in muscle associated with protein loss may cast light on these alternatives. A difficulty with such models is associated with the likelihood that the separation of protein and urea is not complete. The distribution coefficients of urea between cellular and extracellular water are 1.06 and 1.02 in relaxed muscle and muscle in rigor, respectively, indicating that urea is distributed throughout the cell, and therefore also within the soluble protein compartment. The efflux curve for urea would reflect the flux-rate constants for the various compartments. As shown in Fig. 2, the form of the efflux kinetics from muscle equilibrated with urea-C^{14} after full rigor is developed is the same as that of the kinetics from relaxed muscle. A compartment model would have to account for the absence of change in the form of these kinetics following the differential change in the properties of cellular barriers leading to protein loss, while still accounting for the changes in the flux rate of urea which occur before the loss of protein.

In a second explanation, a homogeneous distribution of protein within the cell, but functional differences between the transport of protein and that of urea, would be assumed. Such differences would arise if a change in the state of cellular protein were a prerequisite for its loss. The presence of 1-pentanol might effect such a change, permitting the protein to diffuse from the cell. For example, the protein may not be freely diffusible in the relaxed muscle, but rather adsorbed to the insoluble matrix of the cell. Introduction of 1-pentanol might bring about desorption, the protein then diffusing from the cell through preexisting pores *(10)*. Such a model would have far-reaching implications in the area of cellular lysis *(11)*.

Another type of change in the state of the protein might be a "denaturation" by the 1-pentanol, resulting in a new configuration of the protein permitting it to pass more readily through preexisting pores.

A functional difference between the transport of protein and that of urea may arise in yet another way. Evidence has been presented that the transport of nonelectrolytes, such as urea, is limited by diffusion in the interior of the cell rather than by membrane permeation *(12)*. For example, efflux curves for nonelectrolytes such as those in Fig. 2 show excellent agreement with theoretical curves for diffusion-limited flux. A model of cellular diffusion, based on the assumption of a molecularly ordered protein-water lattice in cytoplasm, has been formulated *(12)*. If urea transport were determined by diffusion and protein were retained in the relaxed cell by a molecular sieve membrane having pores of a size significant for macromolecules but not for small molecules, dissociation of the fluxes of urea and protein could be easily explained. A change-of-state model of protein loss, as considered above, would also be consistent with the diffusion model of urea transport.

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References and Notes

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6. The experimental technique and method of analysis are detailed in I. R. Fenichel and S. B. Horowitz, *Acta Physiol. Scand.* **60**, suppl. 221, ch. 1 (1963). Briefly, a sartorius muscle containing radioactive tracer is stirred in successive baths of Ringer solution containing an equal concentration of unlabeled compound. Analysis of the radioactivity washed out in the baths permits evaluation of the mean tracer concentration $\bar{C}(t)$ in the tissue as a function of time t. The efflux rate constant S_e is the (constant) value of $-$ d ln $\bar{C}(t)/dt$ for large t. In these experiments, urea-C^{14} and 1-pentanol-C^{14} (New England Nuclear Corp.) were used.
7. Measured as the minimum voltage of 0.1-second duration causing contraction. Stimulation was applied with a Grass S4C stimulator and platinum electrodes. Contraction was observed visually.
8. Muscles were kept overnight at 5°C before being washed out. The technique for washing out the muscle was as described in reference *(6)*. The volume of each washout bath was 4 ml, or 5 ml in some experiments. Protein washed out was determined by the Folin method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951). Color development could be abolished by precipitation of the washout solutions with 5 percent trichloroacetic acid and could be recovered by resuspending the precipitate in 0.2*N* NaOH. Bovine serum albumin solutions were used for color standards. A brief period of rapid protein loss at the beginning of stirring in the first bath, similar to that in other "normal" muscle preparations reported in references *(3, 4)*, was invariably seen, but is not shown in Fig. 2.
9. This very slow flux rate has an additional advantage: it makes the contribution of the extracellular space to the flux rate negligible. [I. R. Fenichel and S. B. Horowitz, *Acta Physiol. Scand.* **60**, suppl. 221, ch. 3 (1963)]. The urea flux rate, like the protein loss, is therefore a property of the individual fibers.
10. The complementary proposal is that, in the state of rigor, the enlarged pores are not accessible to urea because it becomes tightly "bound" to insoluble cellular components. This, however, implies an increase in the equilibrium concentration of intracellular urea when the rigor occurs. That this does not occur is indicated by the ratios of intracellular to extracellular concentrations of urea

in relaxed muscle and muscle in rigor, given above.

11. Such a mechanism for lysis was one of the alternatives considered by E. Ponder [*Hemolysis and Related Phenomena* (Grune and Stratton, New York, 1948), p. 256]; G. N. Ling [*A Physical Theory of the Living State* (Blaisdell, New York, 1962), p. 481] offers this mechanism as a consequence of the association-induction hypothesis.
12. I. R. Fenichel and S. B. Horowitz, *Acta Physiol. Scand.* **60**, suppl. 221, chs. 2–4 (1963); S. B. Horowitz and I. R. Fenichel, *Ann. N.Y. Acad. Sci.*, in press.
13. Work supported in part by NSF research grant GB 1794 and by PHS research grant GM 11070. We thank D. Ezekiel for helpful discussions.

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Sex Chromatin in Newborns: Presumptive Evidence for External Factors in Human Nondisjunction

Abstract. *An incidence of 0.6 percent of sex-chromosome aberrations in newborns was found during a 5-month period, while no aberrations occurred in similar populations before and after this period. Down's syndrome also exhibited an elevated frequency during this same critical interval. A severe rubella epidemic may have influenced this pattern.*

With the development of means for determining chromosomal constitution in any human subject (*1*), a new departure in human genetics was instituted which led to the discovery that chromosomal nondisjunction in man is a common cause of disease. It has been estimated that at least 0.5 percent of all human live births are attended by diseases arising from nondisjunction of the chromosomes, which produces serious metabolic disturbance, often including mental deficiency. In addition, it has been calculated that 10 to 15 percent of all human conceptions may result in spontaneous abortion because of chromosomal abnormalities (*2*), and supporting experimental evidence has been presented (*3*).

The discovery by Barr (*4*) of the existence of nuclear chromatin in somatic cells in a number equal to one less than the total number of X chromosomes made possible rapid and convenient study of the presence of abnormalities in the sex chromosomes, in a fashion applicable to large human populations. Studies carried out in different parts of the world (*5–7*) have revealed frequencies of nondisjunction in the sex chromosomes which, when averaged over both sexes, demonstrated values for different human populations, varying between a minimum of zero (*5*) and a maximum of 0.18 percent (*6*). The seriousness of the diseases resulting from such aberrations, as well as the need to understand the basis for this genetic variability in the human species, makes it necessary to answer the following questions: (i) Are the differences in the frequency of the sex chromosomal anomalies reported by different investigators real—or do they simply reflect fluctuations occasioned by the limited size of the samples utilized, or differences in efficiency of the techniques employed? (ii) If the differences are real, do they imply intrinsic, hereditable differences associated with some or all human genetic constitutions, or do they reflect an action of extraneous factors such as diet, temperature, altitude, or exposure to toxic or infectious agents? Since several human autosome pairs such as 13–15, 21, and 18 also participate appreciably in nondisjunctional processes which result in severe developmental anomalies, a definite possibility exists that these chromosomes may be equally susceptible to whatever factors cause nondisjunction in the sex chromosomes, so that the seriousness of the health problem may be large indeed.

A study was undertaken to determine the status of the sex chromatin in somatic cells of the newborn population in the Colorado General Hospital and the General Rose Memorial Hospital in Denver. Details of the mounting, fixation, and staining procedures are described elsewhere (*8, 9*). The sampling in each hospital included all of the babies born on that day. The number of samplings gradually increased to include virtually every baby born on weekdays in both hospitals. The samples were taken by one person and mounted on serially numbered microscope slides, and the frequency of single (and double, if any) chromatin-positive cells was scored by another person who was unacquainted with the identities or phenotypic sex distribution of the babies. Only clear, unwrinkled, well-stained cells were scored, and at least 200 cells were read on each slide. The resulting determination of sex chromatin condition was completed and compared with the phenotypic sex of the baby within 48 hours after birth, so that whenever an unreadable slide or a discrepancy between the sex chromatin status and the phenotypic sex was obtained, additional studies, including full chromosomal analysis and physical examination of the infant, were performed.

During the first 12 months of this study, sex chromatin determinations were carried out by means of buccal smears alone. Approximately 15 percent of the slides so prepared were classified as unreadable because of gross bacterial contamination, poor staining, or an insufficient number of undamaged cells. However, when such samples were repeated on the same babies, clear results were always obtained. In contrast to reports from other laboratories (*10*), the frequency of chromatin-positive cells found in buccal smears of newborn females treated by our procedure was found to be 26 ± 5 percent, a value not significantly different from that found in older females (*8*). Males of any age in no case demonstrated a frequency greater than 3 percent.

The practice was adopted whereby no attempt was made to assess the sex chromatin condition of any doubtful sample, but rather to repeat the sampling procedure immediately, so as to obtain an unequivocal slide. In 1.8 percent of the cases such resampling was impossible because the baby had already left the hospital. Such slides were discarded, unless there was even a suggestion on the original slide of an anomaly. In the latter cases the baby in question was followed up and a clear determination was obtained. By all indications, unreadable samples were due only to technical reasons. A test of the reliability of the method showed the frequency of error in a first reading of 700 slides to be 0.28 percent.

After 1475 cases had been studied, the technique was modified to utilize a sex chromatin determination on the amniotic membrane (*11*). Amniotic preparations differ from buccal smears in displaying a fraction of chromatin-positive cells in normal females of 91 ± 4 percent, instead of only 26 percent, while the frequency in cells from males is still zero (*12*). Consequently, scoring is faster when amniotic membranes are utilized, and marginal mosaics of certain kinds (such as XX/XO) can more readily be detected. The amniotic procedure has the important advantage of making it possible to obtain placentas routinely from the hospital delivery room without disturbing the newly born infant. Enough amnion is available to