nae (Centre National de la Recherche Scientifique, Paris, 1958), vol. 1; the second volume is promised for 1966. A popularized account of coelacanths in given by J. L. B. Smith, *The Search Beneath the Sea* (Holt, New York, 1956). In their paper on lipids of the coelacanth, J. C. Nevenzel, W. Rodegker, J. F. Mead, M. S. Gordon, *Science* **152**, 1753 (1966), provide references to the meager biochemical studies performed on coelacanth tissues.
2. J. Z. Young, *The Life of Vertebrates* (Oxford Univ. Press, London, 1950).
3. We are indebted to Dr. K. S. Thomson and the Peabody Museum of Natural History, Yale University, for making liver tissue available to us through Drs. Clement L. Markert and Edward J. Massaro. This is the same specimen described by Thomson in Ref. *1* (male; 107 cm in standard length; 15.9 kg fresh weight; age about 8 years; caught off Iconi, Grand Comore, 14 March 1966, and frozen immediately). (Catalog No. 1482, Fish Collection, Peabody Museum of Natural History.)
4. H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.* **210**, 33 (1932).
5. H. W. Smith, *Biol. Rev. Cambridge Phil. Soc.* **11**, 49 (1936); E. Baldwin, *ibid.*, p. 247.
6. H. W. Smith, *J. Biol. Chem.* **88**, 97 (1930).
7. M. S. Gordon, K. Schmidt-Nielsen, H. M. Kelley, *Fed. Proc.* **20**, 208 (1961); ——— *J. Exp. Biol.* **38**, 659 (1961); K. Schmidt-Nielsen and P. Lee, *J. Exp. Biol.* **39**, 167 (1962).
8. G. W. Brown, Jr., and P. P. Cohen, *Biochem. J.* **75**, 82 (1960); P. P. Cohen and G. W. Brown, Jr., in *Comparative Biochemistry*, M. Florkin and H. S. Mason, Eds. (Academic Press, New York, 1960), vol. 2, p. 161.
9. G. W. Brown, Jr., *Science* **153**, 1653 (1966).
10. R. M. Archibald *J. Biol. Chem.* **157**, 507 (1945).
11. Enzyme Commission, *Enzyme Nomenclature* (Elsevier, New York, 1965), numbers: urease (E.C. 3.5.1.5), same as urea amidohydrolase; allantoinase (E.C. 3.5.2.5), same as allantoin amidohydrolase; allantoicase (E.C. 3.5.3.4), same as allantoate amidinohydrolase; arginase (E.C. 3.5.3.1), same as L-arginine amidinohydrolase; ornithine carbamoyltransferase (E.C. 2.1.3.3), same as carbamoylphosphate: L-ornithine carbamoyltransferase; formerly also termed ornithine transcarbamylase (*8*).
12. Sigma Type V urease, Sigma Chemical Co., St. Louis, Mo. Lot 30B-647-3.
13. R. J. Block, E. L. Durrum, G. Zweig, *Paper Chromatography and Paper Electrophoresis* (Academic Press, New York, ed. 2, 1958), p. 347.
14. St. J. Przylecki, *Arch. Intern. Physiol.* **26**, 33 (1926); A. Brunel, *Bull. Soc. Chim. Biol.* **19**, 805 (1937).
15. G. Duchateau, M. Florkin, G. Frappez, *Compt. Rend. Soc. Biol.* **134**, 115 (1940); M. Florkin and G. Duchateau, *Arch. Intern. Physiol.* **53**, 267 (1943); R. P. Forster and L. Goldstein, *Science* **153**, 1650 (1966); G. W. Brown, Jr., J. James, R. J. Henderson, W. N. Thomas, R. O. Robinson, A. L. Thompson E. Brown, S. G. Brown, *ibid.*, p. 1653.
16. St. J. Przylecki, *Arch. Intern. Physiol.* **24**, 317 (1924); R. Fosse, A. Brunel, P. de Graeve, *Compt. Rend. Acad. Sci.* **190**, 79 (1930); R. Truszkowski and C. Goldmanówna, *Biochem. J.* **27**, 612 (1933); H. A. Krebs and H. Weil, in *Problems of Biology and Medicine*, O. V. Leontovych, Ed. (Moscow, U.S.S.R., 1935), p. 497 (in German); A. Brunel, *Bull. Soc. Chim. Biol.* **19**, 1683 (1937).
17. H. A. Krebs and L. V. Eggleston, *Ann. Acad. Sci. Fennicae, Ser. A. II Chemica* **60**, 496 (1955), appear to be the first to have reported ornithine carbamoyltransferase (*11*) activity in an elasmobranch (*Scylliorhinus canicula*); see also E. Baldwin, *Comp. Biochem. Physiol.* **1**, 24 (1960); G. W. Brown, Jr., in *International Conference on Taxonomic Biochemistry, Physiology, and Serology*, (Univ. of Kansas, Lawrence, 4–6 Sept. 1962). abstr. 11; G. W. Brown, Jr., in *Taxonomic Biochemistry, Physiology, and Serology*, C. A. Leone, Ed. (Ronald Press, New York, 1964), p. 407; P. P. Cohen and G. W. Brown, Jr., *Proc. Int. Congr. Biochem. 5th Moscow 1961* **3**, 129 (1963); R. L. Joseph, D. C. Watts, E. Baldwin, *Comp. Biochem. Physiol.* **11**, 119 (1964); D. C. Watts and R. L. Watts, *ibid.* **17**, 385 (1966); J. M. Schooler, L. Goldstein, S. C. Hartman, R. P. Forster, *ibid.* **18**, 271 (1966). The assay by Krebs and Eggleston for the enzyme was in reverse of the physiological direction for the synthesis of urea by the ornithine-urea cycle, and they concluded that it represented "a step in the formation of urea in selachian liver." Carbamoylphosphate, chemically synthesized [M. E. Jones, L. Spector, F. Lipmann, *J. Amer. Chem. Soc.* **77**, 819 (1955)], was shown to be the intermediate in the citrulline-synthesizing system of S. Grisolia and P. P. Cohen, *J. Biol. Chem.* **204**, 753 (1953); and R. O. Marshall, L. M. Hall, P. P. Cohen, *Biochim. Biophys. Acta* **17**, 279 (1955). For lungfish see P. A. Janssens, *Comp. Biochem. Physiol.* **11**, 105 (1964); G. W. Brown, Jr., *Science* **149**, 1515 (1965); P. A. Janssens and P. P. Cohen, *Science* **152**, 358 (1966). For Amphibia see G. W. Brown, Jr., in *Physiology of the Amphibia*, J. A. Moore, Ed. (Academic Press, New York, 1964), p. 1; H. Manderscheid, *Biochem. Z.* **263**, 245 (1933).
18. Arginase and ornithine carbamoyltransferase were assayed as described by G. W. Brown, Jr., and P. P. Cohen, *J. Biol. Chem.* **234**, 1769 (1959). Protein was determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. A. Hunter and J. A. Dauphinee, *Proc. Roy. Soc. Ser. B* **97**, 227 (1924).
20. G. W. Brown, Jr., unpublished observations.
21. G. E. Pickford and F. B. Grant, *Science*, this issue.
22. R. M. Archibald, *J. Biol. Chem.* **156**, 121 (1944).
23. A. S. Romer, *The Vertebrate Body* (Saunders, Philadelphia, ed. 2, 1955).

17 November 1966

Nitrate Ions: Potentiation of Increased Permeability to Sugar Associated with Muscle Contraction

Abstract. *Nitrate ions potentiate twitch tension and enhance the increase in permeability to sugar which occurs in electrically stimulated frog sartorius muscles. However, the potentiating effect of nitrate ions on permeability is not dependent upon an increase in twitch tension. The possible relation of changes in permeability to alterations of the concentration of calcium ions in the cell is discussed.*

Exercise is accompanied in vivo by an increased uptake of sugar by skeletal muscles of rats and dogs (*1*). Changes in permeability to sugar have recently been examined in greater detail in frog sartorius muscles stimulated to contract under various conditions in vitro (*2*). Since nitrate ions augment the strength of contraction of muscles in vitro (*3*) we wondered whether or not these ions would modify the increase in permeability to sugar that is associated with contraction.

We used as frog-Ringer bicarbonate solution a modified (*4*) Krebs-Henseleit solution; in nitrate Ringer's solution, $NaNO_3$ was substituted for all of the NaCl. Muscles were stimulated to contract isometrically in Ringer's solution at 19°C with supramaximal, rectangular, direct-current shocks; the initial rate of penetration of 3-*O*-methyl-D-glucose-H^3 was then measured at 19°C as described previously (*2*, *5*) at a substrate concentration of 8 m*M*. 3-Methylglucose, a nonutilizable sugar, and glucose appear to penetrate the muscle cell membrane by way of the same transport system (*5*, *6*). Permeability to sugar can be measured after stimulation is stopped because the effect persists for several hours (*2*).

When frog sartorius muscles are stimulated at frequencies ranging from 3 to 20 shocks per minute, permeability to sugar gradually increases with time and then levels off at a value proportional to the frequency of stimulation (*2*). In our studies, stimulation of muscles in regular Ringer's solution at 15 shocks per minute produced an increase in permeability that eventually leveled off at a value less than the maximum effect attainable with more intense stimulation. When paired muscles were stimulated at the same frequency in nitrate Ringer's solution, permeability rose more rapidly and attained a significantly higher final value (Fig. 1). Thus, nitrate ions can potentiate the effect of electrical stimulation on membrane permeability to sugar.

Nitrate ions had no effect on permeability to sugar in unstimulated muscles. Moreover, when muscles immersed in regular Ringer's solution were stimulated for 30 minutes at a frequency of 120 shocks per minute, a maximum increase in permeability occurred, and nitrate had no further effect.

The rate of penetration of sugar was 11.7 ± 0.8 μmole per milliliter of cell water per hour (mean ± standard error of the mean) for four muscles stimulated in regular Ringer's solution; the rate for paired muscles stimulated in the presence of nitrate ions was 12.3 ± 0.4. The initial rate of penetration of 3-methylglucose into

muscles that have been stimulated in regular Ringer's solution follows saturation kinetics (*2*). These findings suggest that there is a limited number of sites at which stimulation can influence the transport of sugar across the plasma membrane and that nitrate ions modify the effect of stimulation without exerting an independent effect on the membrane.

In the experiments described above, the muscles were supported at a resting tension of 2 g. The peak tension observed during isometric contractions (at a frequency of 15 per minute) was approximately 70 percent greater in nitrate Ringer's than in regular Ringer's solution. The question arose of whether the greater change in permeability observed in muscles stimulated in the presence of nitrate ions was related to the higher tension developed by these muscles. We tested this possibility by decreasing the resting tension of muscles to be stimulated in nitrate Ringer's solution so that these muscles developed a lower maximum twitch tension than the control muscles (Table 1). Despite their weaker contractions, the muscles immersed in nitrate Ringer's solution still showed a greater increase in permeability to sugar than did paired muscles stimulated in regular Ringer's solution.

Table 1. Effect of nitrate on permeability to sugar at a lower twitch tension. One muscle from each frog was stimulated for 30 minutes in regular Ringer's solution at a frequency of 15 stocks per minute after adjustment of the resting tension to 2 g. Paired muscles were stimulated in nitrate Ringer's solution after the resting tension was decreased so that these muscles developed a lower maximum twitch tension than the controls. Permeability is expressed as v, the initial rate of penetration of 3-methylglucose, in micromoles per milliliter of cell water per hour. Each value is a mean for four muscles.

Incubation medium	Peak twitch tension (g)	v (μmole/ml per hour)
Regular Ringer	27.8 ± 0.7	3.0 ± 0.5
Nitrate Ringer	15.8 ± 0.7	5.9 ± 0.4

The potentiating effect of nitrate ions on muscle contraction has been discussed in a review by Sandow (*7*). Although the mechanism of this effect is not yet fully understood, Ebashi *et al.* (*8*) have reported that nitrate ions can impair the active accumulation of Ca^{2+} by vesicles of sarcoplasmic reticulum in vitro. If nitrate can act similarly in intact cells, then the release of Ca^{2+} might be facilitated and the concentration of free Ca^{2+} in the myoplasm might remain elevated for a longer time after excitation.

According to current theories of muscle contraction (*9*), excitation causes depolarization of the cell membrane, which is followed by a release of Ca^{2+} into the myoplasm surrounding the myofibrils; the local increase in concentration of Ca^{2+} is believed to initiate contraction, which is intimately associated with a splitting of adenosine triphosphate. Previous studies (*4*) suggested that, of these events, the one most directly related to the increased permeability to sugar is the increase in concentration of Ca^{2+} in the myoplasm.

Calcium-45 added to the bathing medium enters frog sartorius muscles in appreciable quantities during contractures induced by high concentrations of K^+, and the amount that enters increases as the external concentration of Ca^{45} is raised (*4, 10*). Physiologically significant amounts of calcium ions seem to enter the cells during a potassium contracture since tension remains elevated longer when there are higher concentrations of Ca^{2+} in the medium (*4, 10, 11*). When muscles are exposed to potassium Ringer's solution (in which all of the Na^+ has been replaced by K^+) for a constant, brief interval, an increase in permeability to sugar occurs (*4*); raising the concentration of extracellular Ca^{2+} augments this change in permeability.

Whereas the quantity of adenosine triphosphate and creatine phosphate broken down during muscle contraction is related to the amount of work performed (*2, 12*), the increase in permeability to sugar that occurs after a given number of isotonic contractions is not affected by the work load that is imposed (*2*).

It has been postulated (*4*) that an increase in concentration of Ca^{2+} in the myoplasm initiates two separate processes: (i) contraction and (ii) an increase in the permeability of the cell membrane to sugar. According to this hypothesis the alteration in permeability is not mediated by the interaction of actin and myosin filaments or by closely allied events such as the hydrolysis of adenosine triphosphate. The increase in permeability appears, rather, to be an independent consequence of the change in the myoplasmic concentration of Ca^{2+}. The results of our experiments

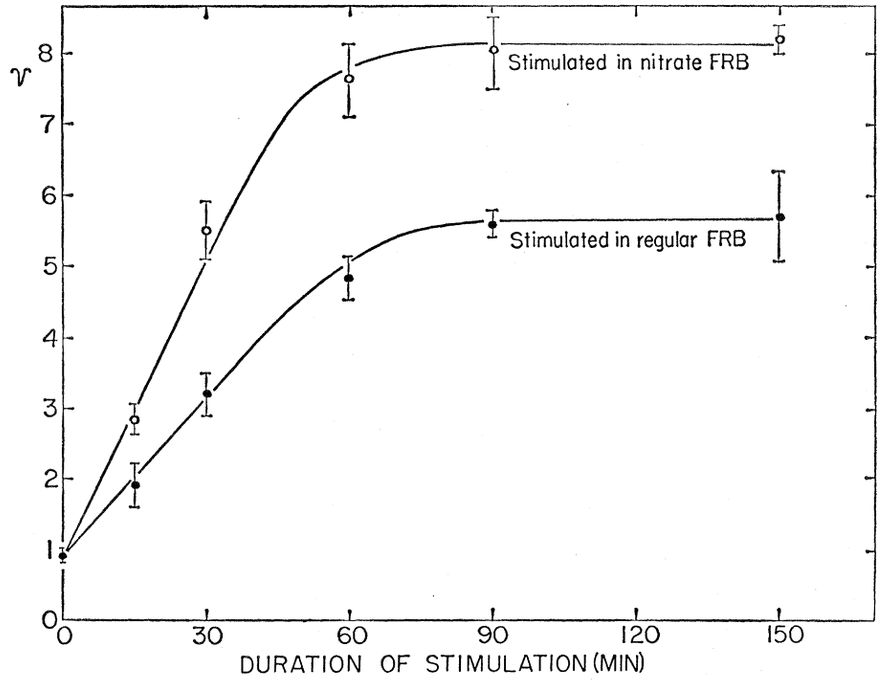

Fig. 1. Effect of nitrate ions on the increase in permeability to sugar caused by stimulation. One set of muscles was stimulated in regular frog-Ringer bicarbonate (FRB) solution, and paired muscles were stimulated in nitrate FRB. Values for v, the initial rate of penetration of 3-methylglucose measured immediately after stimulation, are expressed as micromoles of sugar penetrating per milliliter of intracellular water per hour. Each point is a mean for four muscles, and the vertical bar represents twice the standard error.

with nitrate ion lend further support to this concept.

Morgan *et al.* (*13*) have reported that the permeability of isolated, perfused rat hearts to glucose is enhanced when cardiac output is increased by an increase in the filling pressure of the left atrium. It would be of interest to know whether or not such changes in cardiac output are associated with an altered balance of Ca^{2+} in the heart muscle cells.

JOHN O. HOLLOSZY
H. T. NARAHARA
Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

References and Notes

1. M. S. Goldstein, V. Mullick, B. Huddlestun, R. Levine, *Amer. J. Physiol.* **173**, 212 (1953); E. Helmreich and C. F. Cori, *J. Biol. Chem.* **224**, 663 (1957); W. E. Dulin and J. J. Clark, *Diabetes* **10**, 289 (1961).
2. J. O. Holloszy and H. T. Narahara, *J. Biol. Chem.* **240**, 3493 (1965).
3. A. J. Kahn and A. Sandow, *Science* **112**, 647 (1950).
4. J. O. Holloszy and H. T. Narahara, *J. Gen. Physiol.* **50**, 551 (1967).
5. H. T. Narahara and P. Özand, *J. Biol. Chem.* **238**, 40 (1963).
6. H. E. Morgan and C. R. Park, *Fed. Proc.* **17**, 278 (1958).
7. A. Sandow, *Pharmacol. Rev.* **17**, 265 (1965).
8. S. Ebashi, M. Otsuka, M. Endo, *Proc. Intern Cong. Physiol. 22nd* (1962), p. 899.
9. A. F. Huxley, *Annu. Rev. Physiol.* **26**, 131 (1964).
10. H. Lorković *Amer. J. Physiol* **202**, 440 (1962).
11. G. B. Frank, *J. Physiol.* **151**, 518 (1960).
12. D. F. Cain, A. A. Infante, R. E. Davies, *Nature* **196**, 214 (1962); F. D. Carlson, D. J. Hardy, D. R. Wilkie, *J. Gen. Physiol.* **46**, 851 (1963).
13. H. E. Morgan, J. R. Neely, J. P. Brineaux, C. R. Park, in *Control of Energy Metabolism*, B. Chance, R. W. Estabrook, J. R. Williamson, Eds. (Academic Press, New York, 1965), p. 347.
14. This work was done while Dr. Holloszy was a special research fellow of the National Institute of Arthritis and Metabolic Diseases. The work was supported by NIH grant AM 04082-05 and by a grant from the American Cancer Society to Washington University, St. Louis, Missouri.

17 November 1966

Genomic Exclusion: A Rapid Means for Inducing Homozygous Diploid Lines in Tetrahymena pyriformis, Syngen 1

Abstract. *Genomic exclusion is an abnormal form of conjugation occurring between cells with defective micronuclei and normal cells with diploid micronuclei. The progeny are heterocaryons; each cell has an old macronucleus but a new diploid micronucleus derived from one meiotic product of the normal mate. Such cells express genes found in the old macronucleus, are sexually mature, and can be specifically selected. When inbred, they give rise to lines genetically homozygous at all known loci.*

Few species of ciliated protozoa have been successfully employed in both biochemical and genetic research (*1*). *Tetrahymena pyriformis* is a favorite organism for biochemical experimentation (*2*), since it can be grown on defined medium (*3*) and propagated for many fissions without becoming senile (*4*). Rarely, however, has this experimentation been coupled to genetic analysis. This state of affairs is due to the use of asexual strains of *T. pyriformis*, which lack a micronucleus, and to a reluctance to use the known sexual strains because their maintenance and the techniques of crossing are relatively complex. A chief barrier to their use has been the lack of a rapid method for inducing homozygous diploid lines. Autogamy, a form of self-fertilization, does not take place in this organism; only conjugation, or cross-fertilization, occurs. Thus, genetic testing has necessitated making outcrosses followed by the tedious and time-consuming process of extracting homozygotes by inbreeding. Although genomic exclusion was revealed by the appearance of distorted genetic ratios in crosses of certain inbred strains of syngen 1 (*5*), only recently has the cytogenetic basis of this abnormal form of conjugation been understood (*6*). With proper manipulation, genomic exclusion can serve as a useful tool in the rapid genesis of pure homozygous diploid cell lines. The use of such lines should increase the scope of the biochemical experimentation that is possible with this organism.

For genomic exclusion to occur, one of the two parents must have a defective micronucleus (*7*). In my study a normal clone from the heterozygous AB strain was crossed to a defective clone C* from the inbred C strain. Stained preparations (*8*) of dividing cells showed a normal diploid micronucleus in AB cells (Fig. 1a), while C* cells were hypodiploid (Fig. 1b) or had no micronucleus at all (*9*). In the cross of AB and C*, meiosis was abortive in the C* conjugant (Fig. 1c), and genes were not transmitted from C* to the progeny (*5*).

The sequence of nuclear events that takes place during genomic exclusion was determined by cytological observation and by genetic tests of sample pairs obtained from timed matings of the AB and C* clones. Control crosses used two micronucleate clones from inbred strains A and B. Cultures grown in 1 percent proteose-peptone or in Cerophyl ryegrass inoculated with *Aerobacter aerogenes* were washed in Dryl's physiological salt solution and then mixed. Mating occurred after about 1½ hours. In some experiments, the formation of new pairs was stopped after a specific interval by the addition of nutrient. Samples of pairs were taken periodically for cytological examination (*8*), or single pairs were isolated in Cerophyl-*Aerobacter* medium, and genetic tests were conducted.

A difference in mating behavior was observed during genomic exclusion compared with normal conjugation, if mating was not stopped by the addition of nutrient (Fig. 2). The formation of newly paired cells was followed. In the normal cross (A × B) the frequency of new pairs fell to a constant value between 18 and 36 hours. In the AB × C* cross this frequency fell to 2.5 percent at 16 hours, rose to a second peak of 45 percent at 24 hours, and then fell to 4.2 percent at 36 hours. The timing of the second peak is significant in that it occurred after 12 to 16 hours, a time interval sufficient for completion of normal conjugation. This observation suggested that during genomic exclusion a second mating takes place upon completion of a first mating. The finding that both conjugants had diploid micronuclei in newly paired cells during the second peak (Fig. 1d) strengthened this hypothesis for reasons stated below. The formation of such pairs could be prevented if nutrient was added soon after the parental cultures were mated.

The two consecutive conjugations have been designated Round 1 and Round 2. Round 1 is abnormal. Meiosis is abortive in the C* conjugant. The AB conjugant contributes a single meiotic product, which divides mitotically and gives rise to sister haploid nuclei, one of which migrates to the C* conjugant. Diploidy is reestablished in both mates, probably by endoreduplication, although this point has yet to be documented (*6*). The