brate enzymes, the DNA polymerases of the insects vary. The DNA polymerase-α in the silk glands of silkworms seems to be a great deal larger than the vertebrate enzyme (Fig. 1C), whereas the *Drosophila* embryo DNA polymerase-α is about the same size as the vertebrate enzyme. No *N*-ethylmaleimide–resistant DNA polymerase activity is found in insect tissue extracts. However, there is a low molecular weight, *N*-ethylmaleimide–sensitive DNA polymerase activity amounting to about 1 percent of the total enzyme activity in silk gland extract and about 3 percent of total enzyme activity in *Drosophila* embryo extract. Due to the conditions set for scoring an enzyme as DNA polymerase-β, the low molecular weight enzyme from insects must be scored as negative although it may well be related to the DNA polymerase-β in other eukaryotic organisms. Mixing of silk gland extract and purified calf liver DNA polymerase-β (*4*) prior to dialysis and gradient centrifugation results in complete recovery of calf liver DNA polymerase-β activity in the gradient fraction, indicating that DNA polymerase-β–like activity is not preferentially destroyed in the extract. DNA polymerase-β activity is found in horseshoe crab testes and embryos, but its properties resemble more closely those described for the earthworm. Analysis of DNA polymerase activities in sponge, the simplest of the multicellular animals surveyed here, show DNA polymerase-β activity identical in properties to those found in the vertebrates (Fig. 1D).

The widespread occurrence of DNA polymerase-β in mammals has been noted before (*2*). In an earlier analysis of several fungi (*5*), we were unable to find any activity resembling DNA polymerase-β. No DNA polymerase-β has been reported in protozoa such as *Euglena gracilis* (*6*) and *Tetrahymena pyriformis* (*7*). The enzyme also appears to be absent in fresh extracts of log phase *E. coli* (*8*) cells. There is some controversy about DNA polymerase-β in plant cells, but my analysis of wheat germ and a study of *Vinca rosea* (*9*) indicate little or no activity of this type. The area of principal interest for this discussion then spans the region between the sponges and mammals. DNA polymerase-β is commonly found in representatives throughout this region of biological evolution.

The analyses presented certainly cannot be said to represent all living organisms, and the conditions set for analysis and interpretation are major limitations on detailed arguments about relatedness of any variants noted. Nevertheless, the presence of a well-defined DNA polymerase-β in sponge supports the hypothesis that DNA polymerase-β evolved at the time that animal cells developed a multicellular form. Subsequent evolutionary changes could account for some of the variations in reaction properties observed. The in vivo role of this enzyme in DNA synthesis and DNA repair remains unsolved. Physiological studies have shown that the level of the enzyme remains constant throughout the life cycle of a cell (*2*). The constitutive presence of the enzyme suggests that it has a maintenance function in cellular DNA metabolism. It is of interest that DNA polymerase-β–like enzyme has been conserved over half a billion years of biological evolution, from sponge to man. This observation suggests that the enzyme performs an important function in multicellular animal cells.

LUCY M. S. CHANG
Department of Biochemistry, University of Connecticut Health Center, Farmington 06032

References and Notes

1. A. Weissbach, D. Baltimore, F. J. Bollum, R. Gallo, D. Korn, *Science* **190**, 401 (1975).
2. F. J. Bollum, *Progr. Nucleic Acid Res. Mol. Biol.* **15**, 109 (1974); L. A. Loeb, in *The Enzymes*, P. Boyer, Ed. (Academic Press, New York, 1974), vol. 10, p. 173.
3. T. Kornberg and A. Kornberg, in *ibid.*, p. 119.
4. L. M. S. Chang, *Methods Enzymol.* **29**, 81 (1974).
5. L. W. Loomis, E. F. Rossomando, L. M. S. Chang, manuscript in preparation.
6. A. G. McLannan and H. M. Kerr, *Nucleic Acid Res.* **2**, 223 (1975).
7. M. Creran and R. E. Pearlman, *J. Biol. Chem.* **249**, 3123 (1974).
8. F. J. Bollum, personal communication; L. A. Loeb, *J. Biol. Chem.* **244**, 1672 (1969).
9. J. M. Gardner and C. I. Kado, personal communication.
10. Most organisms used in this study were purchased from Marine Biological Laboratory, Woods Hole, Mass., and Macmillan Science Co., Chicago, Ill. The toadfishes were provided by Dr. D. Watkins, and the silkworms by Dr. J. Steitz.
11. G. Brun, F. Rougeon, M. Lauber, F. Chapeville, *Eur. J. Biochem.* **41**, 241 (1974); L. M. S. Chang, *Fed. Proc.* **34**, 493 (1975); J. G. Stavrianopoulos, J. D. Karkas, E. Chargaff, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2207 (1971).
12. E. Wintersberger, *Eur. J. Biochem.* **50**, 41 (1974).
13. Supported by PHS grants CA 17770 and GM 19789.

14 October 1975; revised 3 December 1975

Sperm Diaphorase: Genetic Polymorphism of a Sperm-Specific Enzyme in Man

Abstract. *Human sperm contains an enzyme with diaphorase activity that appears to be unique to sperm. Electrophoretic analysis of the diaphorase activity in sperm of different individuals reveals three phenotypic patterns. This polymorphism can be explained in terms of two alleles segregating at an autosomal locus; the allele frequencies have been determined to be 0.71 and 0.29. This appears to be the first reported example of a sperm-specific genetic polymorphism in man; its existence raises a number of genetic and biochemical questions.*

Many blood proteins are genetically polymorphic (*1*); less is known about the extent and expression of genetic variation in tissues other than blood. In the course of a study on the expression of genetic variation in human semen, we have discovered an enzyme with diaphorase activity that appears to be both genetically polymorphic and specific to sperm.

The sperm diaphorase polymorphism is readily detected by electrophoresis on polyacrylamide gels (Fig. 1). Sperm extracts from all individuals so far tested exhibit one of the three patterns of diaphorase activity shown in Fig. 1; the pattern type of an individual is persistent over time. The pattern designated type 1 contains a pair of enzyme bands, a and c; pattern type 2 contains bands b and d. All four bands, a, b, c, and d, are present in the pattern type designated 2-1. The composite nature of the type 2-1 pattern was indicated in an experiment in which electrophoresis of a mixture of type 1 and type 2 extracts yielded a pattern indistinguishable from the type 2-1 pattern. These sperm diaphorase patterns are not affected by varying the concentration of sperm extract applied to the gel, nor are they affected by treatment of the sperm extract with reducing agents such as mercaptoethanol.

The individual variation in the electrophoretic patterns of sperm diaphorase is very similar to the electrophoretic patterns given by other enzymes known to be genetically polymorphic (*1*). For example, in the polymorphism at the phosphoglucomutase-1 locus (PGM_1), both homozygote patterns exhibit a pair of enzyme bands, and the PGM_1 heterozygote pattern is the composite of the two homozygote patterns (*2*). By analogy, the electrophoretic variation of sperm diaphorase (SD) may be explained in terms of two allelic genes, SD^1 and SD^2, such that the homozygotes, SD^1SD^1 and SD^2SD^2, give rise to the type 1 and type 2 patterns, respectively; and the SD^1SD^2 heterozygote gives rise to the type 2-1 pattern. The full expression of heterozygosity in males would indicate that the SD locus is autosomal.

Direct demonstration of the heritable basis of this variation has not been achieved because of the difficulty of performing family studies. However, the dis-

tribution of types in the population conforms to the distribution expected, if we assume a genetic polymorphism in Hardy-Weinberg equilibrium (Table 1). For the sample population (N = 52), the calculated allele frequencies are 0.71 and 0.29 for SD^1 and SD^2, respectively. The difference between the observed phenotype distribution and the distribution expected under Hardy-Weinberg equilibrium conditions is not statistically significant (χ^2 for 1 d.f. = 2.326 with Yates' correction for continuity, $.1 < P < .2$). The allelic frequencies of SD^1 and SD^2 place this in the category of a common genetic polymorphism.

Electrophoretic evidence suggests that the polymorphic sperm diaphorase may occur only in sperm and testicular tissue. The diaphorase can be detected in testicular tissue and in sperm collected from vas deferens, as well as in ejaculated sperm. The enzyme is not found in the nonsperm cellular debris of semen (*3*), nor is it found in the seminal plasma of normal or vasectomized males. Moreover, extensive washing of sperm does not remove the diaphorase activity. These observations show that the origin of the sperm diaphorase is the spermatozoon itself. The sperm diaphorase has not been detected in red cells, white cells, heart, liver, or brain. Although extracts of these tissues all have significant diaphorase activity, none of these tissue diaphorases have electrophoretic mobility coincident to that of the sperm diaphorase. Thus sperm diaphorase appears to be specifically limited in its expression to male germ cells.

Diaphorase activity is indicated by the reduction of various dyes with reduced nicotinamide dinucleotide (NADH) or the reduced dinucleotide phosphate (NADPH) serving as electron donors. Diaphorase activity is associated with flavoenzymes; and, not uncommonly, the diaphorase activity of a flavoenzyme has been characterized before the true biological substrate has been discovered (*4*). In this spirit, we have initiated a characterization of the sperm diaphorase activity. We have used an assay in which 25 μl of sperm lysate (prepared as described in the legend to Fig. 1) is assayed in a total volume of 0.5 ml containing 0.05M phosphate (pH 7.0), 0.1 percent bovine serum albumin, 45 μM dichlorophenol-indophenol (DCIP), and 0.67 mM NADH; the reduction of DCIP is monitored spectrophotometrically at 600 nm. The dye, DCIP, is the preferred electron acceptor; the sperm diaphorase appears to have no cytochrome c reductase activity and does not give reproducible assays with potassium ferricyanide as the electron acceptor.

The enzyme has a pH optimum between 7.0 and 7.5, which corresponds to the pH range of human semen (*5*). Unlike most NADH and NADPH diaphorases, the sperm diaphorase accepts both NADH and NADPH as electron donors; the rate of the reaction with NADPH is 76 percent that of the reaction with NADH. That the same sperm diaphorase enzyme accepts both coenzymes was shown by substituting NADPH for NADH in the electrophoresis gel staining mixture. Relatively few of the known diaphorases described are equally active with both NADH and NADPH (*6*). Of those that are active with both coenzymes, several, notably the DT-diaphorases (E.C. 1.6.99.2) from liver, are inhibited by dicoumarol (*6*). The sperm diaphorase activity is not affected by dicoumarol, indicating that it is distinct from the class of diaphorases sensitive to dicoumarol. Thus this enzymological characterization indicates that the catalytic properties of the sperm diaphorase are unlike those described for other diaphorases in other tissues. Our findings lend support to the electrophoretic evidence that sperm diaphorase is unique to male germ cells.

Table 1. Distribution of sperm diaphorase types. Semen samples were obtained from normal volunteers (N = 38) and from patients attending a fertility clinic (N = 14); the data for both populations have been pooled (*7*). More than 90 percent of the contributors were Caucasian. Allele frequencies (SD^1 = 0.71, SD^2 = 0.29) were determined by counting the genes observed in the sample population. The expected phenotype distribution was calculated from these allele frequencies assuming Hardy-Weinberg equilibrium.

Phenotype	Observed	Expected
1-1	29	26.2
2-1	16	21.4
2-2	7	4.4

The sperm-specific expression of sperm diaphorase limits the value of this polymorphism for population genetic studies. However, the sperm diaphorase variation has potential as an individualizing marker in the analysis of semen in the investigation of sex crimes and may be useful in genetic studies of infertility (*7*). Specific inhibition of the sperm diaphorase offers an avenue to fertility control; the development of antibodies to sperm-specific antigens, such as lactate dehydrogenase X, has been suggested as an approach to contraception (*8*).

The existence of a sperm-specific polymorphism also raises a question of a more fundamental nature. The spermatozoon plays an essential role in the reproductive process. Hence, one might predict that sperm would be subject to strong selection pressures, and, on the basis of this prediction, one might expect little genetic variation among sperm proteins and enzymes. However, in fact, at least eight common genetic polymorphisms are expressed in sperm (*9*); the sperm diaphorase polymorphism adds to this number. That genetic polymorphisms are maintained in sperm suggests either that selection effects on different phenotypes are in some way balanced or that the selection differentials on reproductive fitness are negligible. Whether the sperm diaphorase polymorphism is in selective balance or is selectively neutral must await further study.

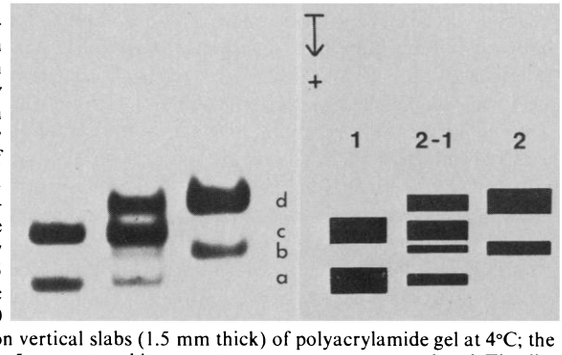

Fig. 1. Electrophoretic patterns given by sperm diaphorase. Sperm were collected from whole human semen by centrifugation at 12,000g for 10 minutes at 4°C. Sperm from a single ejaculate were lysed by freezing and thawing in 0.1 ml of distilled water; alternatively, frozen sperm pellets could have been extracted with water. Lysates were separated from sperm debris by centrifugation at 34,000g for 15 minutes at 4°C. Electrophoretic separation of the diaphorases in 20 μl of sperm lysate was performed on vertical slabs (1.5 mm thick) of polyacrylamide gel at 4°C; the concentration of the running gel was 5 percent, and it was not necessary to use a sample gel. The discontinuous buffer system of Ornstein and Davis (*10*) was used; gels were exposed to a 30-ma constant current until the bromphenol blue marker dye reached the bottom of the gel. The diaphorase stain contained 0.23 mg of dichlorophenol-indophenol (DCIP), 10 mg of NADH, and 5 mg of MTT tetrazolium (Sigma) in 20 ml of 0.25M tris-HCl, pH 8.4; the sperm diaphorase pattern was usually apparent within 15 to 30 minutes after staining at room temperature. Sperm diaphorase also stains if DCIP is omitted from the staining solution but at a much slower rate (*11*).

KEVIN CALDWELL
Department of Genetics, University of California, Berkeley 94720
EDWARD T. BLAKE, G. F. SENSABAUGH
Forensic Science Program, School of Criminology, and *School of Public Health, University of California, Berkeley*

References and Notes

1. H. Harris, *Principles of Human Biochemical Genetics* (North-Holland, Amsterdam, 1971); E. R. Giblett, *Genetic Markers in Human Blood* (Davis, Philadelphia, 1969); H. Harris and D. A. Hopkinson, *Ann. Hum. Genet.* **36**, 9 (1972).
2. N. Spencer, D. Hopkinson, H. Harris, *Nature (London)* **204**, 742 (1964).

3. R. F. Mayol and D. E. Longenecker, *Proc. Soc. Exp. Biol. Med.* **149**, 64 (1975).
4. F. B. Straub, *Biochem. J.* **33**, 787 (1939); V. Masey, *Biochim. Biophys. Acta* **37**, 314 (1960).
5. T. Mann, *Biochemistry of Semen and of the Male Reproductive Tract* (Wiley, New York, 1964), p. 88.
6. L. Earnster, L. Danielson, M. Ljunggren, *Biochim. Biophys. Acta* **58**, 171 (1962); T. E. Conover and L. Earnster, *ibid.*, p. 189; W. R. Frisell, J. R. Cronin, C. G. Mackenzie, in *Flavins and Flavoproteins*, E. C. Slater, Ed. (Elsevier, New York, 1966), p. 367.
7. The distribution of sperm diaphorase types in the fertility clinic patient population differed from that of the normal population; the SD^1 gene frequency was higher in the patient population. It is difficult to assess the significance of this difference, given the relatively small size of the sample population; further population studies are needed to determine whether the difference is real or due to sampling error.
8. R. Stambaugh, B. G. Brackett, L. Mastroinni, *J. Reprod. Fertil.* **19**, 423 (1969); L. J. D. Zaneveld, R. T. Robertson, M. Kessler, W. L. Williams, *ibid.* **25**, 387 (1971); C. B. Metz, *Fed. Proc.* **32**, 2057 (1973); T. S. Li, *Obstet. Gynecol.* **44**, 607 (1974).
9. The expression of the phosphoglucomutase and 6-phosphogluconate dehydrogenase polymorphisms in sperm has been described by W. Renninger and D. Sina [*Humangenetik* **10**, 85 (1970)] and B. Brinkman and E. Koops [*ibid.* **14**, 78 (1971)]. In addition, we have demonstrated the following polymorphic enzymes in sperm: adenylate kinase, esterase D, glucose-6-phosphate dehydrogenase, peptidase A, peptidase C, peptidase D, and phosphoglucose isomerase (E. T. Blake and G. F. Sensabaugh, in preparation).
10. L. Ornstein, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964); B. J. Davis, *ibid.*, p. 404.
11. Sperm contain another enzyme with diaphorase activity which may be visualized on acrylamide gels under special conditions. There is a membrane bound diaphorase that is extracted from sperm by treatment with 1 percent Triton X-100 but is not removed by the freeze-thaw treatment in water; it migrates close to the origin under the electrophoretic conditions described in the legend to Fig. 1.
12. This report is contribution 185 of the Forensic Science Group, University of California, Berkeley. Supported by an Institutional Biomedical Research Support grant from NIH and by grants 74NI-99-0041 and 74NI-99-1007 from the National Institute of Law Enforcement and Criminal Justice, Law Enforcement Assistance Administration, Department of Justice. We thank many colleagues, particularly Drs. A. C. Wilson, R. P. Erickson, and R. Palmour, for advice and comments. Inquiries regarding our work should be directed to G.F.S.

1 October 1975; revised 5 December 1975

Lateral Preoptic Lesions in Rats Separate Urge to Drink from Amount of Water Drunk

Abstract. *Rats with bilaterally symmetrical lesions in the lateral preoptic area do not drink after acute intracellular dehydration, but they drink normally after water deprivation. They, like normal rats, also drink more when cellular dehydration is superimposed upon water deprivation. Unlike normal rats, however, rats with lesions in the lateral preoptic area do not increase their rate of lever-pressing in response to the combined stimulus. Thus, the urge to drink can be separated from the amount of fluid drunk.*

Dehydrating either the cellular (*1*) or the extracellular (*2*) body fluid phases induces drinking. Drinking in response to cellular dehydration terminates upon restoration of the cellular fluid deficit (*1*). Conversely, water intake following extracellular dehydration stops before the deficit is restored (*2*). This is thought to reflect inhibition by cellular overhydration, which results because approximately 70 percent of the ingested water gains access to the cellular phase (*3*). When cellular and extracellular phases are depleted simultaneously, the resulting water intake is a simple addition of the amounts drunk in response to each challenge when presented alone (*4*). Although additivity of drinking is a robust phenomenon, the underlying mechanisms are not known. Understanding this phenomenon is vital for appreciating how behavior contributes to maintenance of normal fluid balance. This is especially important when we consider that, under normal circumstances of fluid abstinence, water is lost from both fluid compartments.

At least three alternative hypotheses can account for drinking additivity. (i) Additivity may reflect an augmented urge to drink so that more water is needed to quench this enhanced thirst. (ii) By virtue of further dehydration of the cellular phase, an inhibitory signal arising from cellular overhydration may be delayed. (iii) There may be a tonic inhibition exerted by the nondehydrated cellular phase over thirst signals arising from extracellular depletion, and dehydrating the cellular phase may relieve this inhibition.

These alternatives cannot be assessed in normal rats which drink in response to both cellular and extracellular dehydration and reduce drinking because of cellular overhydration before restoration of the extracellular phase. Rats with bilaterally symmetrical lesions of the lateral preoptic area (LPO), which do not drink in response to cellular dehydration but are indistinguishable from normal rats in their drinking response to hypovolemia (*5*), are perfectly suited to test these alternatives. We report that the amount drunk by rats with LPO lesions after water deprivation and after water deprivation combined with cellular dehydration is the same as in normal rats, but the rate at which these brain-damaged rats work for water, unlike that of normal rats, is not affected by superimposing cellular dehydration upon a preexisting water deprivation deficit.

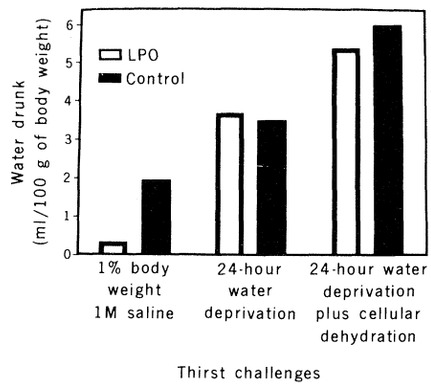

Fig. 1. Amount drunk in 5 hours by rats with LPO lesions and controls after the different thirst challenges.

Ten adult female Sprague-Dawley rats were rendered unresponsive to cellular dehydration by placing bilaterally symmetrical lesions in the LPO (*6*). These rats and an equal number of controls were allowed to drink for 5 hours after acute cellular dehydration (intraperitoneal injections of 1*M* NaCl, 1 percent of body weight), 24 hours of water deprivation, or the combined challenge. As seen in Fig. 1, rats with LPO lesions, which drank nothing or very little in response to the purely cellular stimulus, drank normally after water deprivation and, most importantly, drank as much as normal rats in response to the combined challenge (*7*). These data are not compatible with a theory that volume drunk reflects a simple addition of individual thirsts. However, the results are predicted by an inhibition model, and they suggest that removal or delay of an inhibitory cellular overhydration signal is an adequate explanation for drinking additivity.

Can this reasoning also account for the increased rate of operant responding seen in normal rats with increased dehydration? If increased lever-pressing reflects either a delay of inhibition, resulting from cellular overhydration, or a removal of a tonic inhibition exerted by the cellular phase, then the operant rates of rats with LPO lesions should increase when a cellular dehydration challenge is superimposed upon water deprivation. On the other hand, if the urge to drink mirrors the detection of dehydration of each fluid compartment, then the combined challenge should produce increased response rates in normal rats, which can detect cellular dehydration, but not in rats with LPO lesions, which cannot detect deficits in cellular balance.

Rats with lesions and controls were gradually trained to lever-press on a variable-interval, 30-second schedule of reinforcement when deprived of water for 24 hours. The operant task was specifically designed to reflect changes in the urge to drink independent of satiety and level of intake. Sessions were thus short (30 minutes) and reinforcements were small (0.04 ml); maximum intake per session was lim-