activity. The method employed is not sufficiently sensitive to demonstrate a reduced "reserve" of B_6 in normal gestation, but the data are compatible with the view that pyridoxine supplementation is desirable in human pregnancy.

1. Sprince, H., Lowy, R. S., and Folsome, C. E., *Am. J. Obst. and Gynec.*, 1951, v62, 84.
2. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1952, v40, 1.
3. ———, *Am. J. Obst. and Gynec.*, 1953, v66, 1207.
4. Greenberg, L. D., Bohr, D. F., McGrath, H., and Rinehart, J. F., *Arch. Biochem.*, 1949, v21, 237.
5. Rabinowitz, J. C., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 235.
6. Wachstein, M., and Gudaitis, A., *J. Lab. and Clin. Med.*, 1953, v42, 98.
7. Vilter, R. W., Biehl, J. P., Mueller, J. F., and Friedman, B. I., *Fed. Proc.*, 1954, v13, 776.
8. Snell, E. S., *Physiol. Rev.*, 1953, v33, 509.
9. Marsh, M. E., Greenberg, L. D., and Rinehart, J. F., *J. Nutrition*, 1955, v56, 115.
10. Tonhazy, N. E., White, N. G., and Umbreit, W. W., *Arch. Biochem.*, 1950, v28, 36.
11. Karmen, A., Wroblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.
12. Booher, L. E., and Behan, I. T., *J. Nutrition*, 1949, v39, 495.
13. Beaton, J. R., Beare, J. L., Beaton, G. H., Caldwell, E. F., Ozawa, G., and McHenry, E. W., *J. Biol. Chem.*, 1954, v207, 385.

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Effect of 2,4-Dinitrophenol on Sea-Urchin Sperm. (21930)

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2,4-Dinitrophenol (DNP) has a similar effect on many metabolic systems(1), stimulating respiration and inhibiting energy-requiring processes in low concentrations and inhibiting respiration as well as endergonic processes in high concentrations. Lardy and Phillips(2) showed that DNP stimulates respiration of bull sperm and reduces sperm motility. Since metabolism of sea-urchin sperm differs in several respects from that of mammalian sperm(3,4), it was of interest to examine the effect of DNP on sea-urchin sperm. Dinitrophenol stimulates respiratory rate and causes a reversible block to cleavage in sea-urchin eggs(5,6), but no investigations have been reported concerning the effect of DNP on sea-urchin sperm. Sperm of sea urchins and other animals exhibit "Dilution Effect" (4), characterized by rapid decrease in length of motile life and fertilizing capacity of sperm, and by temporary rise in respiratory rate, upon dilution of the suspension, the rate then falling off toward zero as sperm lose their motility. Metal-chelating agents, such as glycine and ethylenediamine tetracetic acid (Versene), can largely abolish the symptoms of the "Dilution Effect"(7-9), *i.e.* they extend the life span of sea-urchin sperm and suppress the temporary rise in respiratory rate, maintaining respiration at a low, relatively steady level.

It was, therefore, of interest to examine the effect of DNP on sea-urchin sperm in the presence of a metal-chelating agent such as Versene.

Materials and methods. Sperm from the sea urchin *Strongylocentrotus purpuratus* were used. Oxygen consumption was measured with the Warburg respirometer. Dry semen was diluted to a concentration of 4×10^8 to 6×10^8 cells per ml with sea water or with 10^{-3}M Versene† in sea water, and 3 ml of these suspensions were placed in the main compartments of 25 ml Warburg flasks; the gas space in the flasks contained air; CO_2 was absorbed with alkali. Experiments were done at 17.8°C. Dinitrophenol (0.3 ml) was added to sperm suspensions from magnetically control-

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† I am indebted to Versenes Inc., Framingham, Mass., for samples.

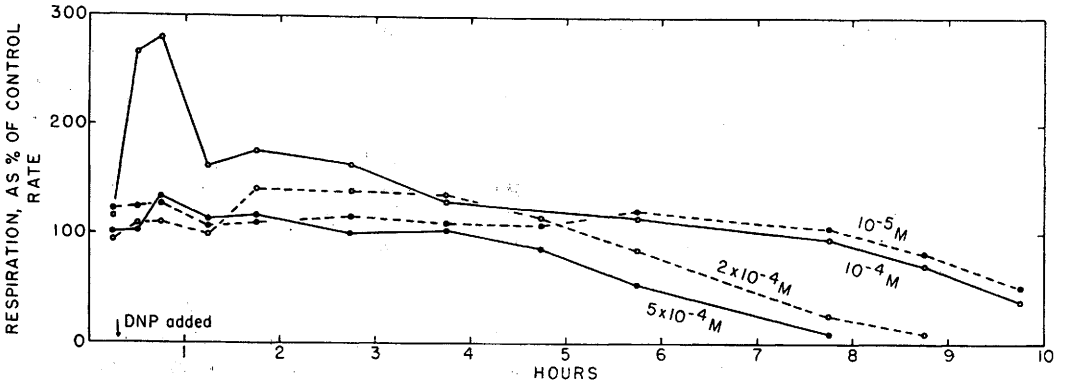

FIG. 1. Effect of DNP on *S. purpuratus* sperm suspended in 10^{-3}M Versene. Curves were determined in separate experiments; respiratory rates were averaged for duplicate flasks.

led cups to give the final concentrations mentioned below The pH of Versene and DNP solutions was adjusted to that of sea water (pH 8.00 to 8.05), and the osmotic pressures were approximately that of sea water. Triplicate test and control flasks were run in each experiment. Two flasks of each set were used for measuring oxygen consumption, and sperm motility observed periodically by removing samples from the third flask.

Results. At 10^{-4}M, 10^{-5}M, and 10^{-6}M, DNP had no appreciable effect on respiration or motility of sperm suspended in sea water. At concentrations between 2 x 10^{-4}M and 5 x 10^{-4}M, DNP inhibited respiration and motility, the degree of inhibition increasing as the concentration of DNP was increased. Inhibition of motility could be reversed by washing and resuspending the treated sperm in fresh sea water.

The response of some systems (including bull sperm) (10) to DNP depends on the initial respiratory rate of the system (1), *i.e.*, the lower the respiratory rate, the more it can be stimulated by DNP. Sea-urchin sperm have a relatively high respiratory rate when suspended in sea water because of the "Dilution Effect." Thus, it seemed possible that if the respiratory symptom of the "Dilution Effect" were suppressed, DNP might then stimulate sperm respiration. Respiration was controlled by suspending the sperm in 10^{-3}M Versene (9), the sperm in this diluent having an initial respiratory rate from 25% to 65% of the initial rate of sperm in sea water.

Sperm in Versene were treated with 10^{-5}M, 10^{-4}M, 2 x 10^{-4}M, and 5 x 10^{-4}M DNP. At 10^{-4}M, DNP initially stimulated respiration to 280% of the control rate (Fig. 1), the respiratory rate of the treated sperm falling below the control rate within 8 hours. The other concentrations of DNP gave less stimulation, and respiration fell below the control rate more quickly as concentration of DNP was increased. Except at 5 x 10^{-4}M, DNP did not detectably inhibit sperm motility until after the respiratory rate of treated sperm fell below that of controls. At 5 x 10^{-4}M, DNP inhibited the motility of Versene-treated sperm by about 40% (visual estimation) within 20 minutes, and the life span of treated sperm was shorter than that of control sperm (Fig. 2).

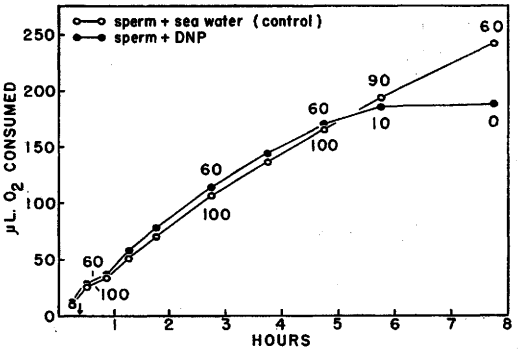

FIG. 2. Inhibition of motility and dissociation of respiration from motility by 5 × 10^{-4}M DNP, sperm in Versene (10^{-3}M). Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 100. Arrow indicates when DNP was added to sperm. (After curves cross, motility ratings are still placed next to curve to which they refer.)

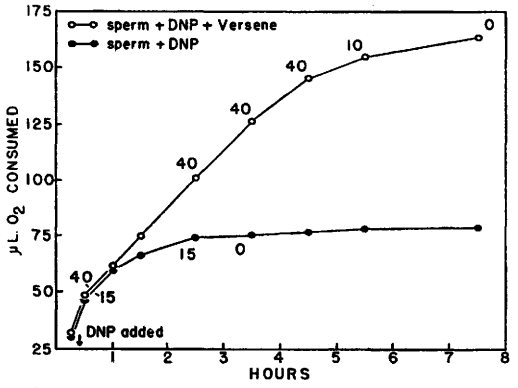

FIG. 3. Protective effect of Versene (10^{-3}M) on *S. purpuratus* sperm in 5×10^{-4}M DNP. Points represent averages for duplicate flasks. Numerals represent motility ratings (visual estimation), initial motility rating = 95.

Versene, as compared to sea water, had a protective effect on sperm treated with 5 x 10^{-4}M DNP (Fig. 3). When DNP was added to sperm in sea water motility was inhibited within 15 minutes, and the sperm rendered completely immotile within 3 hours. Versene, added simultaneously with DNP, reduced the initial inhibition of motility and prolonged the life span and respiration of DNP-treated sperm for 3 to 5 hours.

Unbuffered sea water was used in these experiments, because buffers that might have been used, such as glycylglycine(11), would be likely to affect respiration by virtue of chelating or other specific effects. As a result, the pH of the sperm suspensions in the flasks increased with time (as sperm respiration declines and less respiratory CO_2 is produced, the KOH in the alkali cup begins to extract CO_2 from the sea water diluent). Since DNP penetrates cells more easily as an undissociated molecule than as an ion(12), pH changes alter the effective concentration of the inhibitor. In these experiments, however, the pH of the sperm suspensions in the Warburg flasks did not change appreciably for 1 to 3 hours, indicating that the differential effect of DNP in the presence and absence of Versene was not due to differences in pH of the suspending media.

Discussion. Dinitrophenol at low concentrations can inhibit energy-requiring processes by blocking oxidative phosphorylation and thereby reducing the amount of adenosine-triphosphate available to the cell(13). Since respiration is stimulated as this inhibition occurs, DNP, in effect, "uncouples" respiration from endergonic metabolism. Dinitrophenol apparently effects some "uncoupling" of respiration and motility with *S. purpuratus* sperm. Thus, at 5 x 10^{-4}M DNP inhibits motility while slightly stimulating respiration (Fig. 2). At lower concentrations, however, DNP does not inhibit motility as it stimulates respiration, but "uncoupling" occurs in the sense that the increase in respiratory rate is not accompanied by an increase in sperm activity.

In addition to inhibiting oxidative phosphorylation, DNP also stimulates the adenosine triphosphatase (ATPase) activity of some tissues(14), the two effects sometimes being interrelated(13). It is interesting, then, that Versene and DNP have the same qualitative effect on the activity of rat-brain ATPase(15) as they have on the respiration of sea-urchin sperm. Thus, DNP stimulates the ATPase activity of rat-brain homogenates, but only after the initially high activity of the preparations has been reduced by treatment with ethylenediamine tetracetate.

The effect of DNP on respiration and motility of sea-urchin sperm may involve both an inhibition of oxidative phosphorylation and a stimulation of sperm ATPases.

It is apparent that DNP and dilution have similar effects on *S. purpuratus* sperm. Both shorten the life span and stimulate respiration of sperm, DNP not having a stimulatory effect until that of dilution has been abolished by Versene. Also, both seemingly cause some dissociation of respiration and motility; for dilution this is suggested by the fact that Versene reduces the high respiratory rate of diluted sperm without reducing sperm motility.

As the concentration of DNP is increased, DNP and the agent responsible for the "Dilution Effect" seem to have an accumulative inhibitory effect on sperm respiration and motility.

These similarities raise the possibility that the effects of dilution and DNP occur through similar mechanisms. Thus the "Dilution Effect" may involve an inhibition of oxidative phosphorylation or an increase in the activity

of a sperm ATPase, perhaps one not associated with motility.

Summary. Low concentrations of DNP did not stimulate respiration or affect motility of sea-urchin sperm suspended in sea water; high concentrations of DNP inhibited sperm respiration and motility, the inhibition of motility being reversible. Dinitrophenol stimulated the respiratory rate of sperm in the presence of Versene and caused respiration to be partially dissociated from motility. Versene, as compared with sea water, prolonged the motile life and respiration of sperm treated with high concentrations of DNP. Dinitrophenol and dilution have similar effects on sea-urchin sperm, raising the possibility that both act through similar mechanisms.

1. Simon, E. W., *Biol. Rev.*, 1953, v28, 453.
2. Lardy, H. A., and Phillips, P. H., *J. Biol. Chem*, 1943, v149, 177.
3. Mann, T., *The Biochemistry of Semen*, Methuen and Co., London, 1954.
4. Rothschild, Lord, *Biol. Rev.*, 1951, v26, 1.
5. Clowes, G. H. A., and Krahl, M. E., *J. Gen. Physiol.*, 1936, v20, 145.
6. Tyler, A., and Horowitz, N. H., *Biol. Bull.*, 1938, v75, 209.
7. Tyler, A., and Rothschild, Lord, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 52.
8. Tyler, A., *Biol. Bull.*, 1953, v104, 224.
9. Rothschild, Lord, and Tyler, A., *J. Exp. Biol.*, 1954, v31, 252.
10. Melrose, D. R., and Terner, C., *Biochem. J.*, 1953, v53, 296.
11. Tyler, A., and Horowitz, N. H., *Science*, 1932, v86, 85.
12. Tyler, A., *Proc. Nat. Acad. Sci.*, 1937, v23, 369.
13. Hunter, F. E., Chap. 5 in *Symposium on Phosphorus Metabolism*, v1, Johns Hopkins Press, Baltimore, 1951.
14. Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, 1953, v201, 357.
15. Maxwell, R. E., and Nickel, V. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 846.

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Effect of Age on Polysaccharide Composition of Cartilage. (21931)

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Human cartilage has been subjected to chemical analysis by Hass(1) who studied the effect of aging on its composition. His methods for studies of polysaccharide components consisted of the determination of reducing substances and of sulfate in washed cartilage after hydrolysis with 4.2 N HCl. All of the sulfate and one-half of the reducing substances were assumed to be derived from chondroitin-sulfuric acid under these circumstances. As methods for hexosamine, hexose, and uronic acids are now available and have been used in this laboratory for studies of tissue, it is possible to make more specific studies of the polysaccharide moieties of cartilage. The following study was accordingly initiated.

Methods. Costal cartilage samples were obtained at autopsy from patients who had died from a number of different pathological conditions. The cartilage was freed from extraneous tissue, cut in fine pieces with a surgical knife and dried in a vacuum oven at 60°C. The dried cartilage was ground to pass through a 60 mesh screen.

Uronic acid was determined by a method which was modified from the carbazole method of Dische(2). Ten mg samples of cartilage were weighed into glass stoppered test tubes (16 x 150 mm). Ten ml of 85.7% by volume of sulfuric acid (concentrated H_2SO_4; Sp. Gr. 1.84, diluted 6:1 with distilled water) were added and the tubes placed in a boiling water bath for 15 minutes. After cooling 2 ml aliquots were pipetted into each of 3 glass stoppered test tubes. Twelve ml of 85.7% sul-