the higher vertebrates (*9*). The phylogenetic distribution of polyfucose sulfate is somewhat analogous to that of sialic acid. This compound is present in echinoderms, hemichordates, cephalochordates, and vertebrates (*10*), and is produced by the bacterium *Escherichia coli* in the form of a polymer, colominic acid (*11*), but is absent in the intervening phyla (*10*).

Thus, it appears that closely related acid polysaccharides may have had independent biogeneses. Whether chondroitin sulfate is absent in all of the triploblastic coelomates that are possible common ancestors of the mollusks and arthropods and of the echinoderms is not yet known.

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3. The material was hydrolyzed, under the same conditions of temperature and concentration, at a rate approximately 60 percent of that of a sample of chondroitin-6-sulfate isolated from bovine bone.
4. The stability of the sulfate to strong alkali eliminates the possibility of a 1→4 glycosidic linkage [See E. G. V. Percival, *Quart. Rev. Biol.* **3**, 369 (1949)]. The extreme sensitivity of the glycosidic linkage to acid suggests a 1→2 linkage. The L-fucose was isolated and characterized as the 1-methyl-1-phenylhydrazone (m.p. 178.5°) [See E. Percival, in *Methods in Carbohydrate Chemistry*, R. L. Whistler and M. L. Wolfrom, Ed. (Academic Press, New York, 1962), vol. 1, p. 197].
5. *Hippospongia gossypina*, however, contains a highly sulfated polysaccharide with a large proportion of arabinose [R. L. Katzman and R. W. Jeanloz, unpublished data].
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12. We thank W. D. Hartman, Yale University Peabody Museum, for identifying the sponge. *Amino Sugars LXII*, Publication No. 488 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital. Supported by PHS grants AM-03564 and CA-18166-01.

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Thiamine Release from Nerve Membranes by Tetrodotoxin

Abstract. *Tetrodotoxin (3×10^{-8} molar) promoted the release of thiamine from perfused rat and frog nerve preparations in a manner similar to other neuroactive drugs. When the rats were injected with thiamine labeled with sulfur-35, analyses of brain, spinal cord, and sciatic nerve homogenates revealed labeled thiamine in membrane, synaptosomes, and mitochondrial subfractions. However, on incubation of these fractions with tetrodotoxin, thiamine was released only from the membrane fragments.*

Tetrodotoxin, a poison from the Japanese puffer fish, blocks nerve conduction by inhibiting the early inward current of Na^+ (*1*). We present evidence to implicate thiamine as an intermediate in this blocking activity of the poison.

We have previously (*2*) injected ^{35}S-thiamine into either bullfrogs or rats made deficient in thiamine by dietary restriction, subsequently removed either the spinal cord or sciatic nerves, and perfused the preparation with Ringer solution. When a variety of drugs that cause a change in ion movements in nerves was added to the perfusion fluid, radioactive thiamine was released. Thus, acetylcholine ($10^{-6}M$), potassium chloride ($5 \times 10^{-2}M$), ouabain ($10^{-6}M$), and ethylenediaminetetraacetate ($10^{-3}M$) promoted an immediate efflux of the vitamin, whereas choline ($10^{-4}M$) or NaCl ($5 \times 10^{-2}M$) had no effect. However, the most potent agent in releasing thiamine is tetrodotoxin at a concentration of $3 \times 10^{-8}M$ (0.01 μg/ml) (Fig. 1).

We have studied the stoichiometry of this relation with several different concentrations of tetrodotoxin. Scattered results were obtained so that it was impossible to make a definitive statement on the efficacy of the tetrodotoxin-stimulated efflux of thiamine. Nevertheless, while the ratio of the number of moles of tetrodotoxin necessary to release 1 mole of thiamine varied from 2 to 70, with most of the neuroactive drugs that were tested, a ratio of 200 to 400 was observed. The vitamin that was released into the medium (amounting to 0.1 to 1 percent of the total thiamine content of the preparation) was primarily in the form of thiamine monophosphate (TMP) and free thiamine. In the nerve prep-

Table 1. Release of ^{35}S-thiamine from cell subfractions by tetrodotoxin. Fractions were incubated in Krebs-Ringer phosphate buffer, *p*H 7.2, in the presence and absence of tetrodotoxin ($3 \times 10^{-8}M$) for 20 minutes at 37°C. After incubation, samples were centrifuged at 48,000*g* for 60 minutes to deposit the organelle. A portion of the supernatant was then transferred to a vial containing Bray solution, and the radioactivity was determined in a liquid-scintillation spectrometer. Total radioactivity was determined by deproteinization with trichloroacetate.

Cell fraction	Increase over control (%)	Total radioactivity in subfraction (%)
	Brain	
Membrane	65.1	15.6
Synaptosomes	2.3	0.5
Mitochondria	4.2	.9
	Spinal cord	
Membrane	77.3	40.4
Synaptosomes	47.5	10.2
Mitochondria	5.4	1.4
	Sciatic nerve	
Membrane	31.1	13.3
Mitochondria	0	0

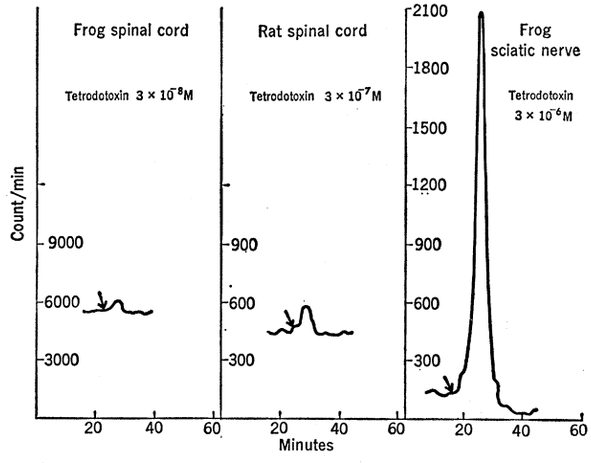

Fig. 1. Effect of tetrodotoxin in promoting the efflux of radioactive thiamine (counts per minute) from perfused preparations. The tetrodotoxin, dissolved in either frog Ringer or Locke solution, was pumped through the nerve chamber at a rate of 1 ml/min for 10 minutes. Parameters of the system were as follows: flow cell volume, 4 ml; rate meter time constant, 40 seconds; chart speed, 7.5 cm/hr. Arrow indicates addition of drug.

aration, the major form of thiamine is thiamine pyrophosphate (TPP) with a small amount of thiamine triphosphate (TTP). This finding suggested an association of ion movements in nerves with dephosphorylation of TPP and TTP.

This study has now been extended to a broken cell preparation. Rats were injected with ^{35}S-thiamine. The brain, spinal cord, and sciatic nerves were then removed and homogenized in 0.32 *M* sucrose. The homogenates were centrifuged at 1000*g* for 11 minutes to remove unbroken cells and nuclei and then at 16,000*g* for 60 minutes to deposit the crude mitochondrial fraction. When these particles, which contained about 70 percent of the total labeled thiamine in the homogenate, were incubated with neuroactive drugs, including tetrodotoxin, radioactive thiamine was released into the medium. The results with both effective and ineffective agents were identical to those of experiments with perfused preparations, except that higher concentrations of the drugs were necessary in this system. To define the organelle from which thiamine was released, this crude mitochondrial fraction was subjected to discontinuous sucrose-density-gradient centrifugation (*3*). With the brain and spinal cord fractions, three distinct protein and radioactivity bands were obtained corresponding to membrane fragments, nerve-ending particles (synaptosomes), and mitochondria; in the sciatic nerve no synaptosomes were observed as would be expected. The purity of the fractions was validated by electron microscopy.

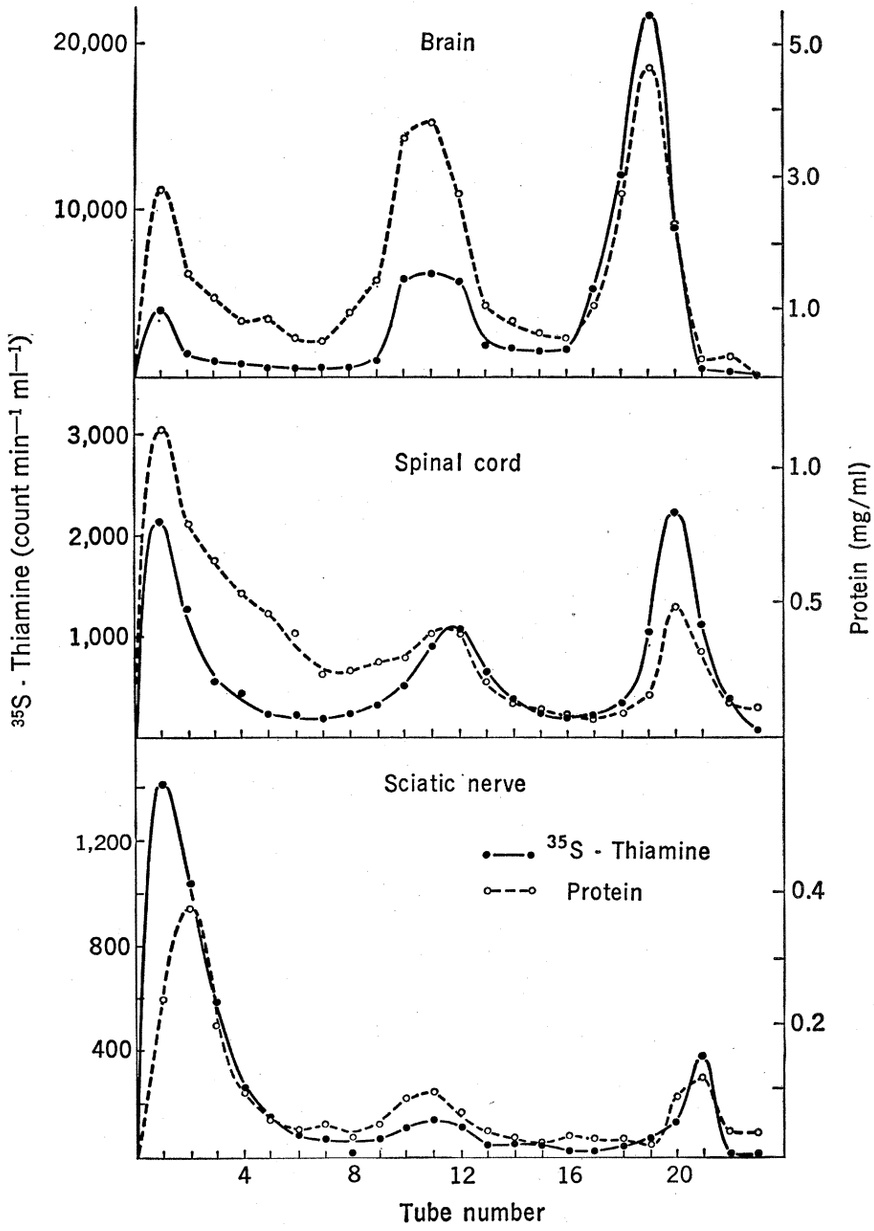

Fig. 2. Protein and radioactive thiamine content of subfractions of rat brain, spinal cord, and sciatic nerve. Tubes are numbered from the top of the gradient. The first peak represents membrane fragments; the second, synaptosomes; and the third, mitochondria.

All fractions contained labeled thiamine (Fig. 2). However, when each of these fractions was incubated with tetrodotoxin we observed specificity of this displacement reaction. Tetrodotoxin released thiamine essentially only from the fraction containing the membrane fragments (Table 1); there was however some release from synaptosomes of spinal cord. In all these release experiments, discharge of thiamine into the medium is artificial because we are dealing with cut surfaces of nerve preparations. In intact tissue the thiamine loss would be expected to be small (*4*).

Our results support the evidence (*5*) that, in nervous tissue, thiamine has a specific role, which is independent of its coenzyme function. The efflux of thiamine from perfused preparations induced by drugs appears to involve the ultimate movement of Na^+. Obviously tetrodotoxin fits into this scheme. This apparent relation between Na^+ and thiamine has already been pointed out by Kunz (*6*). He observed that the antimetabolite pyrithiamine when applied to a single node of Ranvier resulted in an apparent partial inactivation of the Na^+ transport system. We have recently shown that pyrithiamine appears to act by displacing thiamine from nerve preparations (*7*). The propagation of an action potential may involve the shift of TPP or TTP via dephosphorylation from a fixed site in the membrane to allow the early inward current of Na^+. Tetrodotoxin in displacing the thiamine phosphate would occupy the site and prevent the flow of current. The other agents that release thiamine act in a different fashion, perhaps by promoting depolarization.

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Parasexual Cycle in Cultivated Human Somatic Cells

Abstract. *Somatic segregation for three different autosomes was demonstrated in two strains of human diploid fibroblasts derived from subjects known to be heterozygous for chromosomal variants. Recombinant diploid cells appeared within cultures of tetraploid clones isolated from mass cultures. Tetraploid cells regularly occur in mass cultures and within clones of diploid cells. Such a parasexual cycle (2n → 4n → 2n), with recombination of entire linkage groups, could form the basis of a beginning formal genetic analysis in man.*

The discovery of the parasexual cycle of *Aspergillus nidulans* by Pontecorvo and co-workers (*1*) permitted a formal genetic analysis of this and other fungi based entirely on mitotic recombinational events. Pontecorvo suggested that a formal genetic analysis of the human species, based on mitotic recombination within cultivated somatic cells, might also be possible (*1*). The present report (*2*) presents cytogenetic evidence that a parasexual cycle does in fact occur in such cell cultures.

Fibroblast cultures were established from skin biopsy explants derived from a 55-year-old woman, heterozygous for a long submetacentric No. 16 chromosome [the proposita reported in (*3*)], and a 21-month-old girl known to be heterozygous (peripheral blood cultures) for partial deletions of the short arms of chromosome No. 18 and of the short arms of one of the D group chromosomes. Cytogenetic findings in skin fibroblast cultures from the 21-month-old girl were identical to the cytogenetic findings in peripheral blood cultures (Fig. 1). Her father was found to be carrying the D group deletion and her mother, the No. 18 deletion. (Medical genetic aspects of this family will be the subject of a separate report.) The techniques of cell culture, including methods of skin biopsy, cloning, and cytogenectic analysis, have been previously described (*4*).

The frequency of tetraploid cells in mass cultures of these and other strains generally vary from 1 to 4 percent, although in occasional strains frequencies of 10 to 15 percent are observed. One or more tetraploid clones (derived from a single cell as determined by direct microscopy) can readily be isolated from a random sample of 100 cells; therefore, selective techniques were not required to obtain tetraploids. Two such tetraploid clones, one from each of our two chromosomally marked cultures, were examined cytogenetically during repeated "passages" (reseeding of the cultures by trypsinization). The distributions of counts from three such passages gave frequencies of diploid and presumptively diploid cells in the range of 3.7 to 17.1 percent. Comparable proportions of diploid cells have been found in several other tetraploid clones derived from various individuals. There was no evidence of sequential, progressive chromosome loss from tetraploid to diploid. The hypotetraploid counts that were observed (25 out of a total of 171 counts) were interpreted as having resulted from random artifactual losses of chromosomes.

A total of 36 diploid cells were of sufficient quality to permit detailed karyotypes, including group D chromosomes. Of these, 35 were of sufficient quality also to permit complete analysis of the No. 18 chromosome pair. On the basis of visual inspection, four different types of somatic segregants were identified: D/D, Dp−/Dp−, 18/18, and 18p−/18p− (symbol p designates the short arm of the chromosome and the negative sign refers to a deletion) (*5*). These data are summarized in Table 1, and examples of the findings are given in Fig. 1. No instances of double recombinants were found. Twelve recombinants were also found among the 47 diploid cells karyotyped from passages of the other chromosomally marked tetraploid clone (Table 2 and Fig. 2).

Table 1. Cytogenetic characterization of diploid cells from a predominately tetraploid clone of skin fibroblasts from a child heterozygous for partial deletions of two autosomes, one a group D chromosome and the other a group E chromosome (No. 18); p− refers to a short arm deletion.

Karyotypes		No. of cells	Percentage of total cells
Group D karyotypes			
Nonrecombinant	D/Dp−	32	89.0
Recombinant	D/D	2	5.5
Recombinant	Dp−/Dp−	2	5.5
Group E karyotypes			
Nonrecombinant	18/18p−	30	85.7
Recombinant	18/18	4	11.4
Recombinant	18p−/18p−	1	2.9

In order to document our cytogenetic diagnoses, measurements of centromeric indices (ratios of lengths of the short arms to total lengths of chromosomes) (*5*) were determined for all presumed recombinant chromosomes and compared with suitable controls. The results, given in Tables 3 and 4, confirmed the original cytogenetic diagnoses, although the data for recombinants Dp−/Dp− No. 1 and 18/18 No. 1 might be interpreted as having marginal significance.

In order to seek evidence for somatic segregation involving sex chromosomes, 49 diploid cells from two tetraploid clones (derived from a culture made from the foreskin of a newborn) were analyzed. No XX or YY recombinants were found. This is in keeping with Ohno's failure to find examples of somatic segregation involving sex chromosomes in his detailed studies of this phenomenon in deer mice and fish (*6*).

Extensive cytogenetic studies of possible mechanisms of somatic segregation could not be carried out in tetraploid clones from our strains with the autosomal markers because of their eventual

Table 2. Cytogenetic characterization of diploid cells from a predominately tetraploid clone of skin fibroblasts from a woman heterozygous for a morphologic variant of chromosome No. 16; 16?q+ represents a chromosome No. 16 with an apparent increase in length of its long arm.

Group E karyotypes		No. of cells	Percentage of total cells
Nonrecombinant	16/16?q+	35	74.5
Recombinant	16/16	5	10.6
Recombinant	16?q+/16?q+	7	14.9